Food Chemistry 322 (2020) 126710

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidant and anti-inflammatory properties of sourdoughs containing selected Lactobacilli strains are retained in breads

Simone Luti[a,1], Lorenzo Mazzoli[a,1], Matteo Ramazzotti[a], Viola Galli[b], Manuel Venturi[c], Giada Marino[d], Martin Lehmann[d], Simona Guerrini[c], Lisa Granchi[b], Paolo Paoli[a,*], Luigia Pazzagli[a]

[a] Department of Biomedical Experimental and Clinical Sciences, Università di Firenze, viale Morgagni 50, 50134 Firenze, Italy
[b] Department of Agriculture, Food, Environment and Forestry, Università di Firenze, Piazzale delle Cascine, 18, 50144 Firenze, Italy
[c] FoodMicroTeam s.r.l, Via di Santo Spirito n. 14, Florence, Italy
[d] Plant Molecular Biology (Botany), Department Biology I, Ludwig-Maximilians-University München, 82152 Martinsried, Germany

ARTICLE INFO

Keywords:
Sourdough
Bioactive peptides
Antioxidant activity
Anti-inflammatory activity

ABSTRACT

Sourdough fermentation influences several properties of leavened baked goods also because Lactic acid bacteria (LAB) and yeasts produce bioactive peptides with a positive effect on human health. In an early study, three Lactobacilli strains (*L. farciminis* H3 and A11 and *L. sanfranciscensis* I4) possessing different proteolytic activities were used to produce sourdoughs containing peptides equipped with anti-inflammatory and/or antioxidant properties.

This work was aimed to assess whether these properties could be retained after cooking. The selected LABs were used to produce breads from which low molecular weight (LMW-) peptides were extracted. The results provide solid proofs of keeping both antioxidant and anti-inflammatory activities of peptides from cooked products. Sequences of LMW-peptides either from doughs and breads were determined by mass spectrometry: differences have been noticed in amino acidic composition and in sequences, however, all the strains produce peptides equipped with antioxidant and anti-inflammatory activities.

1. Introduction

Bioactive peptides are generally defined as specific protein fragments that may influence the human health by increasing antioxidant and/or anti-inflammatory defence (Chakrabarti, Jahandideh, & Wu, 2014). These peptides generally consist of 3–20 amino acids and may be derived from hydrolysis through chemical or enzymatic treatments of various natural proteins present in different food matrix such as cereals, legumes, milk, eggs, and various marine organisms (Aguilar-Toalá et al., 2017; Chatterjee, Gleddie, & Xiao, 2018; Shadidi & Li, 2015). To date, more than 3600 bioactive peptides from food have been found. The structure/activity relationship among them has been investigated and many of these contain hydrophobic amino acids that seem to account for the antioxidant activity (Zou, He, Li, Tang, & Xia, 2016). Gly, Pro, and Leu, as well as aromatic residues, are among the most frequent ones, and they are frequently structurally organized in random coils (46%) and beta-turns (30%) (Gupta, Sharma, Shastri, Madhu, & Sharma, 2017).

Bioactive peptides produced by chemical or enzymatic cleavage of native proteins have been characterized for the development of nutraceutical or functional foods, but difficulties in setting up of production methods as well as in determining their digestion and adsorption rate have delayed their commercial use (Chakrabarti, Guha, & Majumder, 2018; Giromini, Cheli, Rebucci, & Baldi, 2019). Recent evidence suggests that bioactive peptides can have different biological roles on human health, including antimicrobial, anti-proliferative, antioxidant, blood pressure-lowering, antithrombotic and - stimulating mineral absorption activity (Cicero, Fogacci, & Colletti, 2017). This plethora of features can explain the different roles proposed for bioactive peptides in various pathological conditions such as atherosclerosis, cancer-, and aging-related diseases. Although these pathologies have diverse etiologies, they share many underlying pathological

* Corresponding author.
E-mail addresses: simone.luti@unifi.it (S. Luti), lorenzo.mazzoli@unifi.it (L. Mazzoli), matteo.ramazzotti@unifi.it (M. Ramazzotti), viola.galli@unifi.it (V. Galli), manuel@foodmicroteam.it (M. Venturi), Giada.Marino@biologie.uni-muenchen.de (G. Marino), martin.lehmann@lmu.de (M. Lehmann), simona@foodmicroteam.it (S. Guerrini), lisa.granchi@unifi.it (L. Granchi), paolo.paoli@unifi.it (P. Paoli), luigia.pazzagli@unifi.it (L. Pazzagli).
[1] These two authors contributed equally to the work.

https://doi.org/10.1016/j.foodchem.2020.126710
Received 21 August 2019; Received in revised form 6 February 2020; Accepted 29 March 2020
Available online 03 April 2020

mechanisms including abnormalities in inflammatory responses and oxidative stress (Lugrin, Rosenblatt-Velin, Parapanov, & Liaudet, 2014). In fact, there is an interdependence between oxidative stress and inflammation: oxidative stress can act as inflammation-inducer and lead to different pathologies and further oxidative stress; conversely, inflammation itself can induce oxidative stress, resulting in an increased inflammation response (Biswas, 2016). Based on these evidences, it is reasonable to think that the positive impact of bioactive peptides on human health could be due, almost in part, to their ability to reduce ROS production or to interfere with inflammatory processes.

While polyphenolic compounds have attracted attention for their anti-inflammatory and antioxidant activity for several years (recently reviewed by Costa et al., 2017), the information about the health effects of bioactive peptides is more recent and researches are mainly focused on peptides produced by *in vitro* hydrolysis. However, in recent decades, the consumer interest in functional foods naturally rich in peptides has progressively increased and, consequently, the demand for foods containing fermented products is also increasing (Curiel et al., 2015; Zou et al., 2016). Microbial fermentation, mainly carried out by bacteria belonging to the *Lactobacillus* genus, plays a central role in the production of many foods with functional health properties (Gänzle, 2014; Raveschot et al., 2018). In particular, peptides generated by fermentation of cereals and legumes are of potential importance because they are key components of daily diet all around the world as well as the main component of the Mediterranean diet pyramid (D'Alessandro & De Pergola, 2014). In this respect, sourdough fermentation is the most traditional and effective tool for ensuring rheology, sensory, and shelf-life features, as well as for improving the nutritional value of many food items (Francesca et al., 2019; Gobbetti et al., 2018).

Sourdough is defined as a mixture of water and flour fermented by LAB and yeasts (De Vuyst, Van Kerrebroeck, & Leroy, 2017). In the world, there are hundreds of different sourdoughs, with endogenous microbial community forming a typical microbiota. The metabolism of sourdough microbiota and the activity of cereal enzymes are interdependent, leading to the final product characterized by the accumulation of low-molecular weight compounds, by the degradation of anti-nutritive factors, such as the phytic acid and by the production of various kinds of peptides (Gänzle, Loponen, & Gobbetti, 2008). Small peptides have been isolated following sourdough fermentation of wheat, rye, and quinoa, and their antioxidant and/or anti-inflammatory activity has been determined *in vitro* and in culture cells (Galli et al., 2018).

It is well established that different strains differently hydrolyze cereal proteins, thereby supporting the idea that lactobacilli produce a large number of peptides and highlighting the need to select the best peptide-producing strains (Raveschot et al., 2018; Rizzello et al., 2017). To fulfil this aim, starting from 131 strains of Lactobacilli isolated from Italian sourdoughs, it was possible to identify three strains containing high anti-oxidant and anti-inflammatory activity that was determined as the ability either to reduce reactive oxygen species production or to decrease the expression of some inflammatory markers (Galli et al., 2019). Because of these results encouraged the use of selected starters for the manufacture of healthy leavened bakery products the aim of this work was to assess the retention of anti-oxidant and anti-inflammatory activity in breads manufactured by sourdough containing the selected three LAB strains: *L. farciminis* (A11 and H3) and *L. sanfranciscensis* (I4). Finally, to investigate the amino acid sequences of peptides, mass spectrometry was performed in aqueous extracts from both doughs and cooked goods to unravel the chemical composition of these bioactive peptides.

2. Materials and methods

2.1. Microorganisms growth conditions and enumeration

Four lactic acid bacteria strains were used in this experiment: three lactic acid bacteria strains, L. *farciminis* A11 and H3, and *L. sanfranciscensis* I4, previously characterized and selected for their capacity to increase the antioxidant and anti-inflammatory properties in sourdoughs and the *L. rossiae* O1 for its intermediate biological activity (Galli et al., 2018). The strains were routinely propagated for 24 h at 30 °C in the MR3i medium (Galli et al., 2018). The *Saccharomyces cerevisiae* LV8 strain was aerobically cultured at 30 °C in MYPG (Maltose, Yeast, Peptone, Glucose), a medium containing (in g/L): malt extract 5, yeast extract 3, meat extract 5, and glucose 10. The strains belong to the culture collection of the Department of Agriculture, Food, Environment and Forestry (DAGRI) of the University of Florence (Italy) and were isolated from different Italian sourdoughs. Microorganism enumeration was performed as follow: 10 g of the dough samples were-transferred into 90 mL of a sterile physiological solution and - homogenized for 2 min in a Stomacher Lab Blender 400 (Seward Ltd, Worthing, West Sussex, UK). LABs were diluted and 100 μL of the suspensions were plated on the MR3i medium using the pour plate method and counted after incubation for 48–72 h at 30 °C under anaerobic conditions. *S. cerevisiae* suspensions were plated on the MYPG agar and the colonies counted after incubation for 48 h at 30 °C under aerobic conditions. Plate counts were performed in duplicate.

2.2. Sourdoughs and bread manufacture and water salt soluble extracts (WSE) preparation

Liquid sourdoughs were prepared according to Galli et al. (2018). For bread preparation, cultures of each LAB strain, grown overnight in the MR3i broth, were inoculated at a concentration of ca 10^8 CFU/g, together with a *S. cerevisiae* strain LV8, at a concentration of ca 10^7 CFU/g, into doughs with a dough yield (DY = dough weight × 100/flour weight) of 153. The doughs were prepared by mixing water and wheat flour (Italian milled flour type “00”), and then incubated at 30 °C for 18 h. They were successively subjected to the back-slopping procedure. After the first fermentation, sourdoughs were further propagated (25%, w/w) into fresh flour and water for three times in accordance with the same dough yield. The fermentations were carried out at 30 °C for 6 h. The last back-slopping was used for manufacturing breads, fermented for 4 h at 30 °C and then cooked for 1 h at 180 °C. An acid control (AC) with only *S. cerevisiae* LV8 as inoculum was also prepared with the same recipe and acidified to pH 4.0 through the addition of lactic acid. The water-soluble extracts (WSE) were obtained by extracting from dried breads and liquid sourdoughs with sterile water (1:3 w/V), and then centrifuged at 14,000 × *g* for 20 min at 4 °C.

2.3. Determination of pH, total titratable acidity, and volume

The pH values were determined by a pH-meter (Metrohm Italiana Srl, Varese, Italy). The total titratable acidity (TTA) was measured by using 10 g of dough samples, which were homogenized with 90 mL of distilled water for 3 min and expressed as the amount (mL) of 0.1 N NaOH to achieve a pH of 8.5. To assess the sourdough’s increasing volume, 200 g of each dough was placed in a graduated cylinder (1 L). The volume of the doughs (in mL) was recorded immediately and after 4 h of fermentation at 30 °C before the cooking. The leavening was calculated using the following formula: $[(V_4 - V_0)/V_0] \times 100$, where V_4 was the volume after the 4 h fermentation and V_0 was the initial volume (Zannini et al., 2009).

2.4. Fractionation of water-soluble extracts

Water liquid extracts from liquid sourdoughs and breads were

assayed for protein and peptide content by the bicinchoninic acid method (BCA, Pierce Chemical, Rockford, IL, USA). About 3 mg/mL of each sample was added to 0.05% (v/v) trifluoroacetic acid (TFA), centrifuged at 10,000 × *g* for 10 min and analyzed by the C18-reverse phase HPLC (Thermo Fisher Scientific, MA, USA); column was from Vydac, 4.6 × 250 mm; 5 μm; 300 Å, Columbia, MD Elution was carried out at 0.8 mL/min, using a water/acetonitrile gradient, containing 10 mM trifluoroacetic acid (TFA). Solvent A: H_2O + 10 mM TFA; Solvent B: CH_3CN + 10 mM TFA. Gradient: 0–10 min 30%B, 10–50 min 50%B 50–60 min 100%B. The eluted peptides were fractionated according to the retention times as low molecular weight (LMW-, 5–25 min) and high molecular weight (HMW-, 25–60 min) fractions.

The solvents were removed from collected fractions by freeze-drying and then the LMW fractions were re-dissolved in sterile water to the same concentration for further characterization.

2.5. Cell viability assay

RAW 264.7 murine macrophages (Sigma Aldrich, St. Louis, MO, USA) were cultured in standard conditions (5% CO_2, 37 °C) in Dulbecco's modified Eagle medium (DMEM) and then added with 10% (w/v) fetal bovine serum (FBS), 100 μg/mL penicillin/streptomycin and 1 mM glutamine. The cells were routinely sub-cultured every two days.

The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St Louis, MI, USA) method was used to test the cell viability by evaluating the capacity of succinate dehydrogenase to convert MTT into formazan crystals (Tolosa, Donato, & Gómez-Lechón, 2015). The cells were plated on 24-well plates in 1000 μL of fresh medium at a density of 5 × 10^3 and grown for 24 h. As much as 10 μL of each LMW extracts were added at a final concentration of 0.01 mg/mL and incubated at 37 °C for further 24 h. The cells without the addition of LMW-extracts were used as the negative control; cells treated with 1 μg/mL Lipopolysaccharide (LPS) were used as the positive control. After incubation, 250 μL of 0.5 mg/mL MTT in DMEM without red phenol was added and incubated in the dark at 37 °C for 45 min. Finally, 200 μL of DMSO (dimethylsulfoxide) was added and the absorbance was quantified at 595 nm with a microplate reader (BioRad, Hercules, CA, USA).

2.6. Intracellular reactive oxygen species (ROS) measurement

The cells were treated for 1 h with LMW-extracts at the final concentration of 0.01 mg/mL and then co-incubated with 1 μg/mL of LPS for 24 h. All the stressed cells were used as the positive control, while the cells treated with 0.05 mM of Ascorbic acid were used as the negative control.

The level of intracellular ROS was assessed by measuring the oxidation of the fluorescent probe 2′,7′ –dichlorofluorescin diacetate (25 μM DCFH-DA in DMSO). The probe was added to the medium and the cells were incubated for 1 h. The cells were lysed with the RIPA buffer (50 mM TRIS-HCl, 150 mM NaCl, 100 mM NaF, 2 mM EGTA, 1% Triton X-100) and then centrifuged at 10,000 × *g* for 10 min. Fluorescence was detected at the excitation/emission wavelengths of 485/538 nm using a Fluoroscan Ascent FL microplate fluorescent reader (Thermo Electronic Corporation, MA, USA). The values were determined as fluorescence intensity units and the results expressed as a percentage of the reduction in ROS formation.

2.7. Immunoblot analysis

RAW 264.7 cells (2 × 10^6) were plated in a 6-well multiplate and treated with LMW-extracts and 1 μg/mL of LPS for ROS determination. Positive and negative controls were also prepared treating cells with LPS and with LPS plus 2 mM acetyl salicylic acid, respectively. After 24 h of incubation, the cells were lysed in the Laemmli buffer. Next, 30 μg of each sample was resolved by 10% SDS-PAGE and transferred to PVDF membranes. The membranes were treated with phosphate-buffered saline (PBS), containing 5% bovine serum albumin (BSA) and 0.1% Tween 20, -and they were maintained in incubation for 1 h at room temperature (25° C). Finally the membranes were treated with the primary antibodies—pNFkB 93H1, NFkB D14E12 and IkB 44D4— as markers of the nuclear factor NF-kB pathway (Cell Signaling Technology, Inc. Danvers, MA, USA) and they were left to incubate overnight. Anti-COX2 C-20 (Santa Crux Biotechnology, Dallas, Texas, USA), anti-iNOS, and PA1-036 (Thermo Fisher Scientific, MA, USA) antibodies were used to detect the expression levels of Cyclooxygenase 2 and of Inducible Nitric Oxide Synthase, respectively. Actin (anti-actin13E5 antibodies, Cell Signaling) was used as a reference protein. The antibodies were used diluted 1:1000. The blotted membranes were washed three times and incubated with horseradish peroxidase-conjugated secondary anti-rabbit antibodies (Cell Signaling) diluted 1:2000 in PBS containing 5% BSA and 0.1% Tween 20 for 1 h. After successive washing, the membranes were developed using an ECL kit (GE healthcare). Chemiluminescence signals were acquired with a molecular imaging station from Kodak and the values obtained from the densitometric analysis were normalized on the actin signal.

2.8. LC-MS/MS analysis of LMW fractions

LMW fractions from the acid control (AC) and the samples H3, A11, I4, and O1, from both liquid sourdoughs and breads, were analysed using a high resolution Q-TOF Impact II (Ultra-High Resolution Qq-Time-Of-Flight, Bruker-Daltonik GmbH, Bremen, Germany), coupled to an UltiMate™ 3000 RSLC nano system (Thermo Fisher Scientific, Waltham, MA, USA). As much as 1 μg of each LMW sample was injected twice. The peptides were first loaded into a 2 cm trap column (Acclaim™ PepMap™, C18, 100 Å, 100 μm × 2 cm, ThermoFisher Scientific, Waltham, MA, USA) and then separated by reverse phase chromatography using a 50 cm analytical column (Acclaim™ PepMap™ C-18, 100 Å, 75 μm × 50 cm, ThermoFisher Scientific, Waltham, MA, USA). The flow rate was 250 nL/min and the peptides were eluted by a water/CH_3CN gradient, containing 0.1% formic acid (FA). Solvent A: H_2O + 0.1% FA; Solvent B: CH_3CN + 0.1% FA. Gradient: 0–5 min, 5% B; 5–120 min, 35% B; 120–135 min, 45%B; 135–137 min, 90% B; 137–147 min, 90% B; 147–150 min, 5% B and 150–170 min, 5% B. MS1 spectra were acquired in the *m/z* range 200–2200. The Top 18 most intense peaks were selected and analyzed by MS/MS. To prevent redundant sampling, the selected precursors were excluded from the MS/MS analysis for 30 *sec* after fragmentation.

2.9. Protein identification from MS analysis

Peak lists obtained from the MS/MS spectra and converted into the MGF format were searched using Comet v. 2016.01 rev. 3 within SearchGUI v. 3.2.20 (Eng, Jahan, & Hoopmann, 2013). Protein identification was conducted against a concatenated target/decoy (Vaudel et al., 2015) version of a reference protein database composed by the UniProt sequences from *Triticum aestivum* (146,076 entries), available entries from members of the *Lactobacillus* genus (9916 entries), and known MS contaminants. The decoy sequences were created by reversing the target sequences in SearchGUI. The search was performed using the following parameters: digestion unspecific, fragment ion types by precursor *m/z*, tolerance 20 ppm, fragment *m/z* tolerance 0.1 Da, and precursor charge 1–4 no fixed modifications, variable modifications: oxidation of methionine (+15.994915 Da).

Peptides and proteins were inferred from the spectrum identification results using PeptideShaker version 1.16.15 (Elias & Gygi, 2010). Peptide spectrum matches (PSMs), peptides, and proteins were validated at a confidence of 1.0% False Discovery Rate (FDR) was estimated using the decoy hit distribution. Proteins were considered as identified if at least one confident peptide could be identified by at least one confident spectrum. The mass spectrometry proteomics data has been

deposited to the ProteomeXchange Consortium (Deutsch et al., 2017) with the dataset identifier PXD013454 and https://doi.org//10.6019/PXD013454.

2.10. Creation of an online peptides inventory

Peptides considered of high quality (in terms of the number and quality of PSM) in each sample (H3, A11, O1, I4, and AC) were collected for both cooked and uncooked extracts. FASTA files were created and indexed with appropriate annotations representing unique sequences per condition (for a total of 10 conditions). Such sequence files were pooled and formatted for BLASTp search. A web-server was then designed to interrogate such databases using candidate sequences as queries. The default search parameters were fixed to word size = 2 and scoring matrix PAM30, similar to the configuration of the online NCBI BLAST optimized for short matches. The search engine is freely available at http://bioserver2.sbsc.unifi.it/bioinfo/pazzagli/soursearch.html.

2.11. Post-processing of MS data

Data were analyzed using two Perl script developed in-house. The first script implements a modified method from Secher et al. (2016). The method is inspired by the concept of longest peptide variants (LPV) which is aimed at reducing the complexity of the peptide dataset. Briefly, our algorithm retains the longest peptides, irrespective of the source protein, over the whole peptide set and maintains an accurate spectral abundance. To achieve this, all confidently identified peptides (T) and the count of their PSM are loaded. Afterwards, the peptides are sorted by length and, starting from the longest peptide L, the peptides $T \neq L$ are recursively mapped onto L. During iterations, if the peptide C was found to be perfectly and entirely contained on L, it is removed from the list of available peptides and its number of PSM_c is accumulated onto PSM_L in accordance with (1):

$$PSM_L = \sum_{i=1}^{T} PSM_C \quad (1)$$

where PSM_L is the peptide spectra match of the longest peptide and PSM_c is the PSM of peptides contained in the longest peptide.

The second script is inspired by the coverage plots frequently used in NGS to detect copy number variations. Briefly, given a set of protein sequences of interest, the lists of confidently identified peptides are mapped onto the protein sequence and stacked to compose a position-specific coverage by summing up the number of the PSM of stacking peptides in accordance with (2):

$$COV_p = \sum_{i=1}^{L} PSM_{c,i} \quad (2)$$

where COV_P is the coverage profile of the protein of interest P of length N, and $PSM_{c,i}$ is the number of PSM if the peptide C covers the position i.

Such procedures are implemented as Perl scripts (MS_pept_collapse.pl and MS_prot_covplot.pl) and are freely available at https://github.com/matteoramazzotti/MSutils.

3. Results

3.1. Microbiological and technological properties of sourdough and breads

Four bacterial strains, namely *L. farciminis* A11 and H3, and *L. sanfranciscensis* I4, and *L. rossiae* O1 were used to obtain breads. These strains were chosen for their proven ability to improve anti-oxidant and anti-inflammatory activity of liquid sourdoughs (Galli et al., 2018).

The acidification parameters (pH and TTA) of the bread before cooking were in line with those reported for sourdough breads, showing a pH < 4.5 and a TTA higher than 3.5 mL (Minervini et al., 2018), thus indicating a proper activity of all the LAB strains that reached a final concentration of 9 log CFU/g. The increase in the dough volume was significantly lower in the dough inoculated with *L. sanfranciscensis* I4 and S. *cerevisiae* than in doughs inoculated with *L. farciminis* A11, *L. farciminis* H3 and S. *cerevisiae* or *L. rossiae* O1 and S. *cerevisiae,* respectively. This lower increase of the dough volume was probably due to the competition for maltose utilization between *L. sanfranciscensis* and *S. cerevisiae*. leading to a reduction of yeast growth rate and fermenting activity. Breads after cooking 1 h at 180 °C (Fig. S1) were subjected to an internal sensory analysis, evaluating the general liking (scale 1–9) of the breads. They were all acceptable with average score of 7.

3.2. Fractionation of water-soluble extracts (WSE)

The water-soluble extracts (WSE) obtained from dried breads were fractionated by reverse-phase chromatography to isolate peptides with low molecular weight. One bread defined acid control (AC) that was obtained with only *S. cerevisiae* as inoculum was also fractionated to detect proteolysis due to the lactic acid. As for peptides from sourdoughs (Galli et al., 2018), fractionation was performed on the retention times: low molecular weight (LMW): 5–25 min; and high molecular weight (HMW): 25–60 min. The fractions were collected by hand-picking (Fig. 1). Chromatographic layouts (Fig. 1A,B) show a clear separation between high and low molecular weight peptides that was confirmed by a 15% SDS-PAGE (Fig. S2). The chromatographic layouts of the H3 sample and of the AC were very similar to the I4 and O1 ones (Fig. S3). As observed for WSE from sourdoughs, also the AC one contains many peptides, indicating that the chemical acidification itself is able to induce a proteolytic fragmentation

Hence, all fractions containing the LMW peptides from the cooked samples were collected, lyophilized, and assayed to determine their protein concentration. Then, they were re-suspended at the same concentration and used for detecting biological activity on cultured cells.

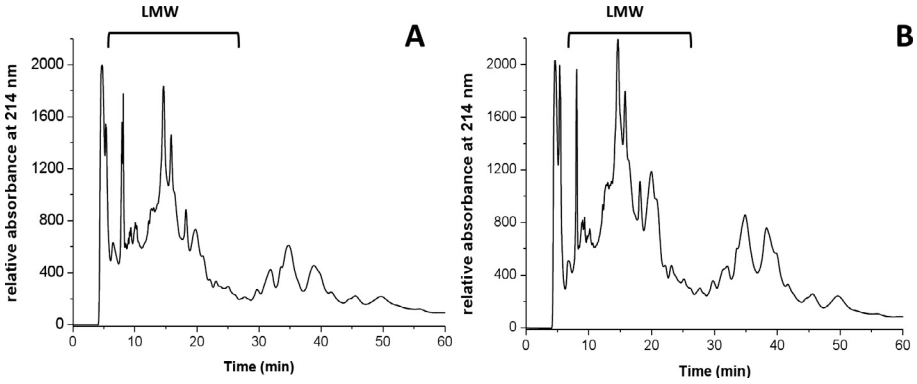

Fig. 1. HPLC fractionation of two representative SWEs from *L. rossiae* O1 strain (A) and *L. farciminis* H3 strain (B). 400 µL aliquot were applied on a Reverse Phase column (Vydac, 4,6 × 250 mm; 5 µm; 300 Å) and eluted with a non-linear gradient (Solvent A: H_2O + 10 mM TFA; Solvent B: CH_3CN + 10 mM TFA. Gradient: 0–10 min 30%B, 10–50 min 50%B 50–60 min 100%B). LMW: collected peptides.

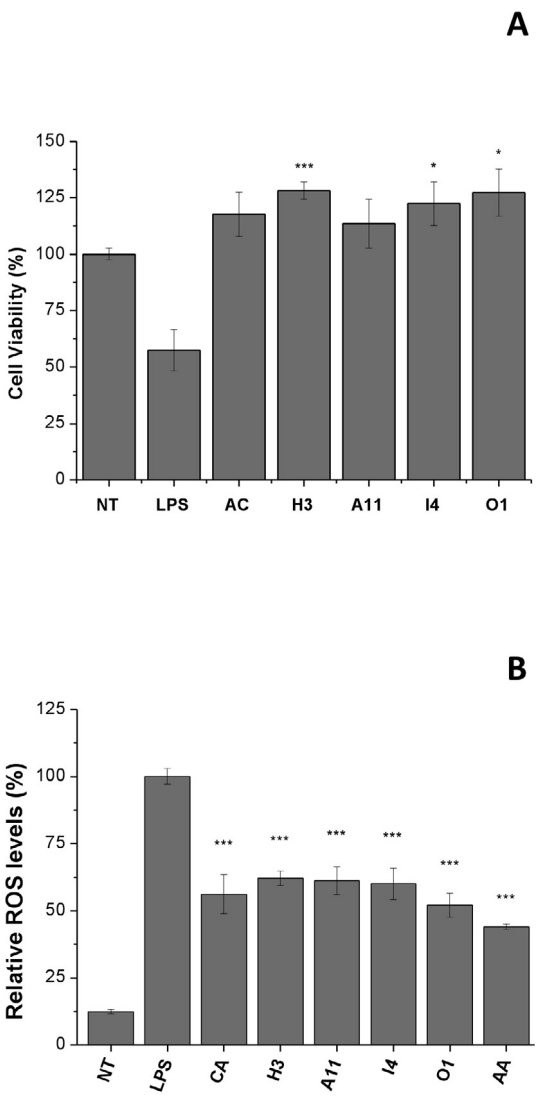

Fig. 2. Effect of LMW fractions on cell viability and ROS production. A) Effects of LMW peptides on cell viability. RAW 264.7 were incubated with LMW-peptides (0.01 mg/ml) or LPS for 24 h. After this time, cell viability was evaluated using MTT test. The absorbance of formazan was determined at 595 nm. NT: Negative Control, untreated cells; LPS: positive control, cells treated with the only LPS; AC, acidic control; H3, A11, I4, and O1 indicate cells treated with LMW peptides obtained from doughs fermented with *L. farciminis* H3 and A11 strains, *L. sanfranciscensis* I4 strain and *L. rossiae* O1 strain, respectively. Data reported in the figure represent the mean (± SD) of three different experiments performed in duplicate. Statistical analysis was performed for each sample vs NT (*p < 0.05; ***p < 0.01). B) Intracellular ROS levels in RAW cells. Cells were treated with 0.01 mg/mL LMW fractions and 1 μg/mL LPS as reported in material and methods section. NT: Negative Control, untreated cells; LPS: positive control, cells treated with the only LPS; AC, H3, A11 I4 and O1 are for LMW peptides of the Acidic Control, *L. farciminis* H3 and A11, *L. sanfranciscensis* I4 and *L. rossiae* O1 strains, respectively; AA: cell treated with 0.05 mM Ascorbic Acid. Results are the mean (± SD) of three different experiments performed in duplicate. Statistical analysis was performed for each sample vs the LPS value (*** p < 0.01).

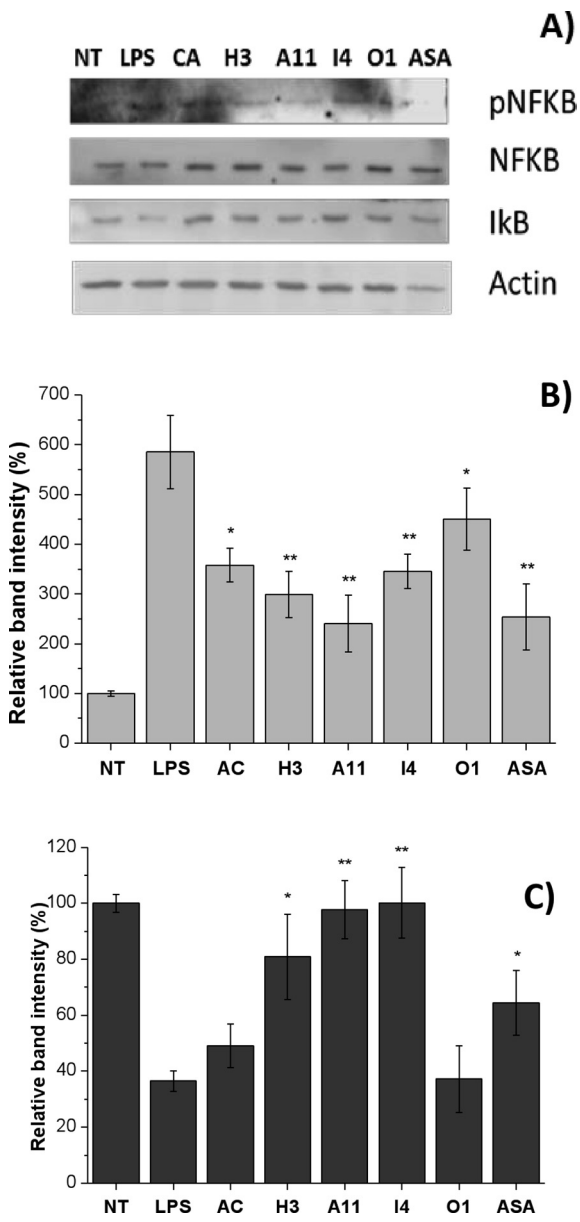

Fig. 3. Western blot analysis of NFkB (p65) and IkB from RAW 264.7 cells. A) Western blot analysis of cells lysates detected by anti-phospho-NFkB p65, anti-total NFkB p65 and, IkB mAbs; B, C) Relative protein levels from Western Blot data. Intensity of each signal was measured: pNFkB/NFkB ratio (B, light gray) and IkB (C, dark gray) are normalized on the actin signal. The intensity of each band was measured by using a Molecular Imaging Station. NT: no treated cells; LPS:, cell treated with lipopolysaccharide; ASA: cell treated with acetyl salicylic acid. AC, H3, A11 I4 and O1 are for LMW of the Acid Control, *L. farciminis* H3 and A11 strains, *L. sanfranciscensis* I4 strain and *L. rossiae* O1 strain, respectively. Results are the mean (± SD) of four different experiments performed in duplicate. Statistical analysis was performed for each sample vs the LPS value (* p < 0.05; ** p < 0.01).

3.3. Cell viability

RAW 264.7 cells (murine macrophages) were used as cellular model to study the biological activity of - fractions containing the LMW peptides. LPS treated cells were chosen as positive control because of LPS ability to induce the activation of inflammatory pathways, to increase the synthesis of reactive oxygen species, and consequently, to reduce cell viability (Pan et al., 2019). The MTT test was performed to evaluate cell viability of macrophages treated with LMW-peptides obtained from breads previously fermented with the selected *Lactobacillus* strains. The total mitochondrial activity detected by the test can be related to the number of viable cells; it can be used to measure the *in vitro* cytotoxic effects of a variety of metabolites (Tolosa et al., 2015). Results show that none of tested samples impairs cell viability, thereby confirming that LMW-peptides are per se not toxic (Fig. 2A). The viability of RAW cells slightly increased in comparison with the non-treated cells, especially for the H3 sample.

3.4. Antioxidant activity of LMW peptides from breads

We wondered whether LMW extracts were able to inhibit ROS production. To answer this question, macrophages were pre-incubated with LMW extracts for 1 h, and then stimulated with LPS for further 24 h to highlight the ability of the extracts to reverse the formation of free radicals induced by LPS itself. For all the analyzed samples, the reduction of free radicals was highly significant, and it was quite similar to those detected in cells treated with ascorbic acid (Fig. 2B). Therefore, these data indicate that the cooking procedure did not affect the biological activity of peptides produced during the fermentation process. The percentage of ROS reduction does not significantly differ from that obtained with LMW peptides from sourdoughs (Galli et al., 2019). Comparing the values obtained with LMW peptides from doughs and breads no significant differences can be detected at $p < 0.05$ (Table S1). Finally, a decrease in intracellular ROS levels was also obtained with LMW fractions from the acid control that contains the peptides obtained by lactic acid addition as observed for sourdough extracts [Table S1; 19].

3.5. Anti-inflammatory activity of LMW extracts from cooked samples

The anti-inflammatory activity of LMW fractions obtained from the breads was determined by detecting the expression levels of some of the main markers involved in the inflammatory response. In particular, the phosphorylation status of NFkB expressed as the ratio between the phosphorylated and total form (pNFkB/NFkB, Gupta et al., 2018), and the expression of its inhibitor, the IkB factor, were determined in the cooked samples as previously performed on LMW extracts from sourdough (Galli et al., 2018). The activated inflammatory factor (pNFkB/NFkB) is important for the transcription of a wide variety of genes involved in the control of the host's immune and inflammatory response (Milanovic, Kracht, & Schmitz, 2014). On the contrary, the expression levels of IkB, the main inhibitor of the activity of NFkB, increase in the presence of anti-inflammatory signals (Gupta et al., 2018; Park et al., 2016). Moreover, iNOS (inducible Nitric Oxide Synthase) and COX2 (Cyclooxygenase, isoenzyme 2) expressions were determined as downstream markers of the anti-inflammatory response.

RAW cells were pre-treated with LMW extracts for an hour and then incubated with LPS for further 24 h before the analysis; the lysates were analyzed by western blot and detected with specific mAbs (Fig. 3A). We found that the ratio pNFkB/NFkB significantly decreased in macrophages treated with LMW extracts. In particular, peptides from the strain A11 showed a potency similar to that of acetylsalicylic acid (Fig. 3B). Moreover, macrophages treated with LMW extracts obtained from strains A11, H3, and I4 expressed levels of IkB just like the one detected in the control sample, thereby confirming that those extracts possess a potent anti-inflammatory activity (Fig. 3C). In agreement with the intermediate activity of peptides derived from O1 sourdough, peptides derived from O1 bread have a less pronounced effect on the NFkB expression, which is, however, significantly different from the LPS expression. Finally, the acid control does not show any increase in IkB expression to suggest that peptides derived by acidification have a less pronounced anti-inflammatory activity. Moreover, comparing the data from both Fig. 3B and C setting the LPS value as 100% (Fig. S4), the decrease in the pNFkB/NFkB value goes together with the increase in the IkB value, as expected for anti-inflammatory compounds. Results clearly indicate that cooking doesn't affect the anti-inflammatory activity of the LMW peptides extracted from breads obtained with the LAB strains previously selected (Galli et al., 2018)

To corroborate the results, the anti-inflammatory activity of extracts from the cooked samples was also detected by analysing the expression levels of iNOS and COX2 as downstream markers of the anti-inflammatory response. iNOS was chosen as one of the key enzymes forming nitric oxide (NO) that play an important role in various pathophysiological conditions, inflammation, infection, and progression

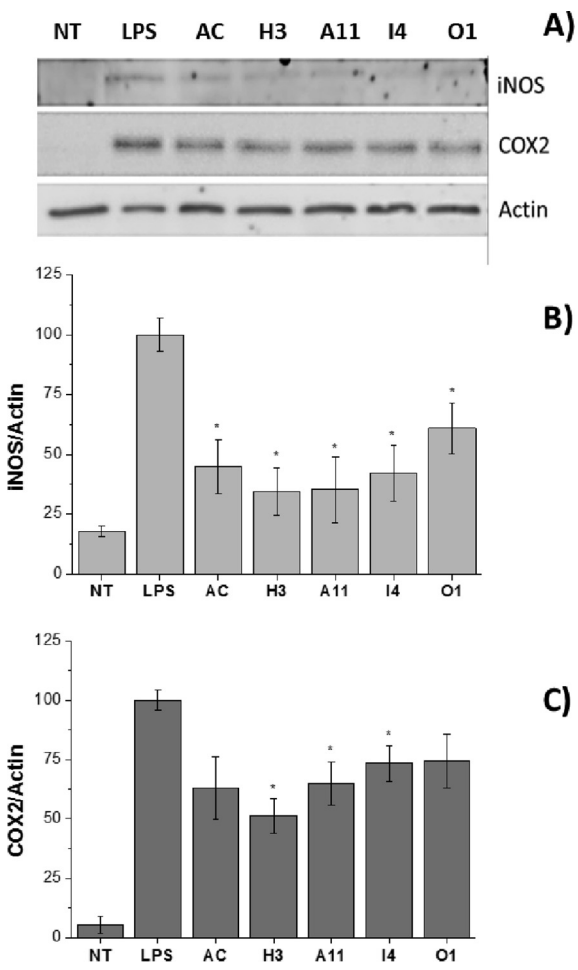

Fig. 4. Western blot analysis of iNOS and COX2 from RAW 264.7 cells. A) Western blot analysis of cells lysates detected by anti-iNOS and anti-COX2 mAbs. The intensity of each band was measured by using a Molecular Imaging Station from Kodak. B, C) Relative protein expression levels obtained from Western Blot data of iNOS and COX2, respectively. NT: Negative Control, untreated cells; LPS: positive control, cells treated with the only LPS; AC, H3, A11 I4 and O1 are for LMW of the Acid Control, *L. farciminis* H3 and A11 strains, *L. sanfranciscensis* I4 strain and *L. rossiae* O1 strain, respectively. Results are the mean (± SD) of three different experiments performed in duplicate. Statistical analysis was performed for each sample vs LPS value (* $p < 0.01$).

of malignant diseases; COX2, also known as Prostaglandin-endoperoxide synthase 2 remains unexpressed under normal conditions and increases during inflammation (Ramyaa, Krishnaswamy, & Padma, 2014; Xue, Yan, Zhang, & Xiong, 2018). Results showed in Fig. 4 demonstrate that all the extracts, including acid control, can reduce the iNOS expression, whereas only H3, A11, and I4 extracts trigger a significant reduction in COX2 expression levels.

3.6. Overview about the biological activity of peptides from breads

The three selected best LAB strains were used to produce sourdoughs and breads from which bioactive peptides can be extracted. To the best of our knowledge, this is the first report highlighting the keeping of biological activity of peptides in cooked food.

To obtain results that could be compared with the ones obtained on sourdough fermentation, both extraction and fractionation procedures were performed in keeping with the procedure proposed by Galli et al. (2018). LMW peptides have been easily recovered after RP-HPLC and then used at 0.01 mg/mL to treat RAW264.7 cells, which are used as a model (Pan et al., 2019). All the analyzed samples are not toxic, and

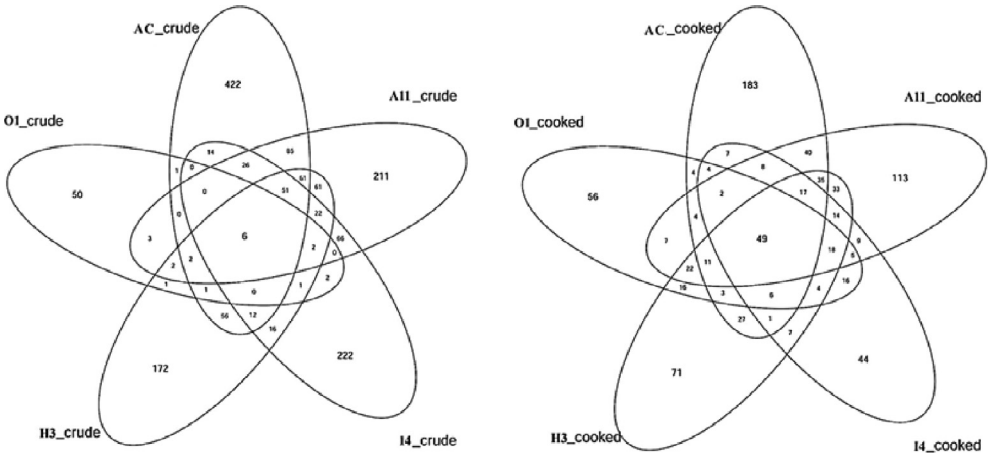

Fig. 5. Venn diagram of LPV (Longest Peptide Variants) in sourdoughs (left panel) and in cooked samples (righ panel). Numbers are referred to the number of peptides in each sample identified by a peptide collapsing algorithm to highlight the LPV (Longest Peptide Variants). AC, H3, A11 I4 and O1 are from the Acidic Control, *L. farciminis* H3 and A11 strains, *L. sanfranciscensis* I4 strain and *L. rossiae* O1 strain, respectively.

peptides from H3, I4, and O1 strains trigger an increase in cell vitality. The results are in agreement with those obtained with peptides from sourdough and suggest a general positive role on cellular health. In fact, by assaying the level of intracellular ROS in cells stressed with LPS and treated with the peptides, anti-oxidant activity is found present and it is within the same range of those obtained in ascorbic acid-treated cells. The anti-oxidant activity is maintained in breads, and it is not different ($p > 0.05$) from the one obtained with LMW peptides derived from the respective crude extracts (Table S1)

However, to demonstrate that the cooking procedure does not impair the biological activity of the product, LMW peptides have been also tested for their ability to counteract the inflammation pathway induced by LPS. In all the samples, a statistically significant decrease in the pNFkB/NFkB expression goes along with a great increase in the IkB expression, thereby corroborating the anti-inflammatory features of LMW peptides from cooked breads. As expected, the A11, H3, and I4 strains trigger a sharpe reduction in COX-2 and iNOS expression. This is in agreement with recent observations that the such expression act in concert with NF-kB activation and IkB degradation on the inflammation pathway (Gupta et al., 2018). Although these activities are also possessed by the acid control, it is important to notice that the decrease in pH values of sourdough, needed to activate endogenous proteases, is obtained by addition of lactic acid giving rise to bread with bad rheology and sensory features.

3.7. Identification of bioactive peptides from sourdoughs and breads by mass spectrometry

Mass spectrometry was performed on LMW peptides from sourdoughs and breads. Peptides have been obtained by aqueous extraction and therefore we could only analyse the hydrophilic ones, which are, however, the most promising for further studies that could determine their role *in vivo* experiments (Chakrabarti et al., 2018).

The analysis was performed and statistically filtered by using the programs SearchGUI and PeptideShaker which allowed to search mass spectra collections versus an *ad-hoc* database of protein sequences (in this case from the proteomes of *Triticum aestivum* and several *Lactobacillus* species, see Material and Methods) as well as to evaluate the significance of peptide-spectra matches against a decoy database (Vaudel et al., 2015). Matches were further refined using a peptide-collapsing algorithm designed to retain the longest of a series of overlapping peptides (LPV, longest peptide variants) without losing their overall intensity values. The most represented LPVs recovered in doughs and breads are reported in Table S2. The most of the overlapping peptides are from 20 to 39 amino acids long. Other shorter peptides are present in the extracts and increase the number of peptides contributing to the biological activity of the fraction. Most of the identified peptides are derived from gliadins, glutenins, serpins, and beta amylase from wheat, thus confirming the previous observation that bioactive peptides are encrypted in inactive precursor protein sequences (Gänzle, 2014; Zou et al., 2016). Moreover, results showed differences in protein target: in extracts from doughs most of the peptides derives from gliadins and serpins, while, in extracts form breads a larger number of peptides is from uncharacterized proteins, aquaporin and beta-amylase. To investigate this more closely, differences between cooked and crude extracts were analyzed according to a procedure called peptide coverage profiling. Despite the huge variability of such proteins in terms of sequence, we were able to identify many peptides corresponding to N- and C-terminal fragments as well as some peptides from internal regions. In glutenins (Fig. S5), acid control preparation was mostly rich in residual peptides, with fragments spanning most of the sequences. In contrast, we observed a lower number of mapping peptides in the LMW extracts obtained by LAB fermentation. The most noticeable examples were the O1 strain with coverage only in the extreme C-terminus and the A11 strain with some coverage also in the central region. When the cooked counterparts were analyzed, a drastic reduction in coverage was observed in terms of mapping the peptides commonly and uniquely being restricted to the C-terminus. In gliadins (Fig. S6), the crude extracts showed a marked mapping on both N- and C-terminals in all the investigated treatments. Different from glutenins, the cooking procedure did not alter the residual peptide coverage profile (Fig. S6).

Venn diagrams were used to summarize the similarities among different datasets in terms of shared peptide sequences and accordingly to their source proteins (Fig. 5). Although a lot of specificities are evident all datasets from the cooked material converged to 49 peptides, while all datasets from the crude material converged to six peptides only (see Table S2 for details). When the most representative peptides in doughs and baked extracts are compared, their differences in terms of the sequence are evident, suggesting that during the cooking procedure temperature may influence the cleavage process. Only three peptides are present in all the analyzed extracts (Table S1), while a large number of peptides are strain-specific, confirming that the hydrolysis of the precursor proteins is a strain-specific feature (Galli et al., 2018; Gänzle, 2014).

The results from the Venn diagram also allow the identification of peptides that are unique for each sample (Table S2). These peptides obviously represent the contribution of the specific *Lactobacillus* strain used to obtain sourdough and the respective bread without adding any chemical reagent. It is noteworthy that extracts from acid control had the highest value of abundance, probably due to the addition of lactic acid, causing a rapid decrease in the pH value and leading to fast activation of proteases from wheat. The lower number of peptides in both sourdoughs and breads from fermentation due to the metabolism of *Lactobacillus* strains is not be excluded. To further analyse differences between crude and cooked samples, we evaluated the amino acid

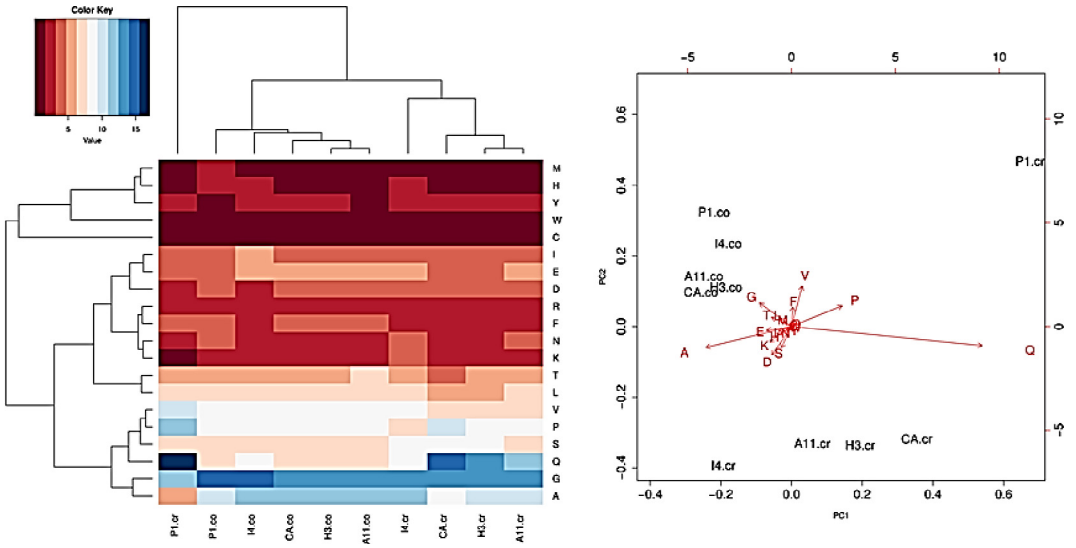

Fig. 6. Multivariate analyses on amino acid composition of identified peptides. Left: heatmap on percent composition of the 20 amino acids. Dendrograms are based on complete clustering of 1-Spearman correlation distances (samples) and Bray-Curtis distance (amino acids). Right: biplot of principal component analysis on the same data.

composition of the peptides identified in different preparations. As shown in the PCA part of Fig. 6, this criterion clearly separates the cooked and crude samples, with the unique exception of O1 crude that is characterized by an unusually high content of Q. Amino acid loadings indicate that a higher proportion of G and V features of the cooked samples, while D and S are (less robust) features of the crude samples. When data is represented in the heat map of Fig. 6, the cooked samples are clearly clustered together with a marked discriminating contribution by Q. Our data indicates that the most abundant amino acids recovered in all the analyzed samples are P, S, Q, G, and A; it partially agrees with the literature where G, P, L, and A are reported as the amino acids that are present in a high percentage in peptides from foods (Zou et al., 2016). Hydrophobicity, another feature of bioactive peptides (Zou et al., 2016), is not evident in our samples, probably because it has not been found due to aqueous extraction. Glutamine is largely present in peptides from doughs, clearly as a consequence of the high content of this amino acid in glutenins and gliadins from which the peptides are derived from. Conversely, a lower content of glutamine is present in peptides from cooked breads, where proline, valine, and leucine are the most frequent amino acids. This analysis reinforced the observation that cooking flour determines a marked alteration of residual peptides, possibly due to an altered contribution of glutenins and gliadins that are particularly rich in glutamine. However, although a look into the peptide data bank as well as the literature does not enable the identification of any consensus sequence to be addressed by some biological activity, many peptides of different lengths are obtained by the enzymatic digestion and fermentation of cereal flour; they show anti-oxidant, anti-inflammatory, and anti-hypertensive activity, but a structure/function relation has not been outlined till now (Cavazos & de Mejia, 2013; Pan et al., 2019; Suetsuna & Chen, 2002; Sun et al., 2019).

4. Conclusion

Although bakery yeast fermentation is the most used production method in industrial bakeries, the interest in sourdough fermentation is rising because of the sensory properties, shelf-life, and improved nutritional value of the products In fact, sourdough fermentation induces changes in metabolites potentially having health-promoting properties and naturally leads to the production of bioactive peptides. Recently, the anti-oxidant and anti-inflammatory properties of some LMW peptides from Italian sourdoughs have been detected on cultured cells: three strains, namely *L. farciminis* H3 and A11 and *L. sanfranciscensis* I4, have been found to be particularly able in producing bioactive peptides Consequently, deeper knowledge of the biological activity and chemical composition of these peptides might be useful for manufacturing new baked products with nutraceutical properties. Therefore, in this study, the three selected LABs have been used to produce breads from which LMW peptides were extracted. As the peptides from doughs, the peptides from breads had the same antioxidant and anti-inflammatory activity that mainly happens by suppressing the NFkB pathway as well as reducing the intracellular ROS levels. This fact complies with the observation that the oxidative response is deeply involved in the regulation of the inflammatory processes and *vice versa*, since NFkB is sensitive to the intracellular redox state and modulated by reactive oxygen species. The differences in sequences and amino acidic compositions between peptides from dough and breads that have been noticed, do not affect the biological activity which is preserved after cooking. Therefore, new baked products could be produced from sourdoughs fermented with one (or more) active *Lactobacillus* strain(s) and their ability to counteract inflammation, and to prevent the onset of chronic degenerative disease such as the irritable bowel syndrome and the development of type 1 diabetes could be studied.

CRediT authorship contribution statement

Simone Luti: Investigation, Data curation. **Lorenzo Mazzoli:** Investigation, Software. **Matteo Ramazzotti:** Software, Formal analysis. **Viola Galli:** Investigation, Validation. **Manuel Venturi:** Investigation, Data curation. **Giada Marino:** Writing - review & editing. **Martin Lehmann:** Writing - review & editing. **Simona Guerrini:** Project administration, Writing - original draft. **Lisa Granchi:** Funding acquisition, Supervision. **Paolo Paoli:** Investigation, Project administration. **Luigia Pazzagli:** Conceptualization, Funding acquisition, Supervision.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgement

This study was supported by the Italian Ministry of Education, University and Research (MIUR) program PRIN 2015 (grant number 2015SSEKFL) “Processing for healthy cereal foods”.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2020.126710.

References

Aguilar-Toalá, J. E., Santiago-López, L., Peres, C. M., Peres, C., Garcia, H. S., Vallejo-Cordoba, B., ... Hernández-Mendoza, A. (2017). Assessment of multifunctional activity of bioactive peptides derived from fermented milk by specific Lactobacillus plantarum strains. *Journal of Dairy Science, 100*(1), 65–75. https://doi.org/10.3168/jds.2016-11846.

Biswas, S. K. (2016). Does the interdependence between oxidative stress and inflammation explain the antioxidant paradox? *Oxidative Medicine and Cellular Longevity, 5698931*. https://doi.org/10.1155/2016/569893.

Cavazos, A., & de Mejia, E. A. (2013). Identification of Bioactive Peptides from Cereal Storage Proteins and Their Potential Role in Prevention of Chronic Diseases. 12, pp. 364–380. https://doi.org/10.1111/1541-4337.12017.

Chakrabarti, S., Jahandideh, F., & Wu, J. (2014). Food-derived bioactive peptides on inflammation and oxidative stress. *Biomed Research International, 608979*. https://doi.org/10.1155/2014/608979.

Chakrabarti, S., Guha, S., & Majumder, K. (2018). Food-derived bioactive peptides in human health: Challenges and opportunities. *Nutrients, 10*, E1738. https://doi.org/10.3390/nu10111738.

Chatterjee, C., Gleddie, S., & Xiao, C. W. (2018). Soybean bioactive peptides and their functional properties. *Nutrients, 10*(9), E1211. https://doi.org/10.3390/nu10091211.

Cicero, A. F. G., Fogacci, F., & Colletti, A. (2017). Potential role of bioactive peptides in prevention and treatment of chronic diseases: A narrative review. *British Jornal of Pharmacology, 174*, 1378–1394. https://doi.org/10.1111/bph.13608.

Costa, C., Tsatsakis, A., Mamoulakis, C., Teodoro, M., Briguglio, G., Caruso, E., ... Fenga, C. (2017). Current evidence on the effect of dietary polyphenols intake on chronic diseases. *Food and Chemical Toxicology, 110*, 286–299. https://doi.org/10.1016/j.fct.2017.10.023.

Curiel, J. A., Coda, R., Centomani, I., Summo, C., Gobbetti, M., & Rizzello, C. G. (2015). Exploitation of the nutritional and functional characteristics of traditional Italian legumes: The potential of sourdough fermentation. *International Journal of Food Microbiology, 196*, 51–61. https://doi.org/10.1016/j.ijfoodmicro.2014.11.032.

D'Alessandro, A., & De Pergola, G. (2014). Mediterranean diet pyramid: A proposal for Italian people. *Nutrients, 10*, 4302–4316. https://doi.org/10.3390/nu6104302.

De Vuyst, L., Van Kerrebroeck, S., & Leroy, F. (2017). Microbial ecology and process technology of sourdough fermentation. *Advances in Applied Microbiology, 100*, 49–160. https://doi.org/10.1016/bs.aambs.2017.02.003.

Deutsch, E. W., Csordas, A., Sun, Z., Jarnuczak, A., Perez-Riverol, Y., Ternent, T., ... Vizcaíno, J. A. (2017). The ProteomeXchange consortium in 2017: Supporting the cultural change in proteomics public data deposition. *Nucleic Acids Research, 54*(D1), D1100–D1106. https://doi.org/10.1093/nar/gkw936.

Elias, J. E., & Gygi, S. P. (2010). Target-decoy search strategy for mass spectrometry-based proteomics. *Methods in Molecular Biology, 604*, 55–71. https://doi.org/10.1007/978-1-60761-444-9_5.

Eng, J. K., Jahan, T. A., & Hoopmann, M. R. (2013). Comet: An open-source MS/MS sequence database search tool. *Proteomics, 13*(1), 22–24. https://doi.org/10.1002/pmic.201200439.

Francesca, N., Gaglio, R., Alfonzo, A., Corona, O., Moschetti, G., & Settanni, L. (2019). Characteristics of sourdoughs and baked pizzas as affected by starter culture inoculums. *International Journal of Food Microbiology, 293*, 114–123. https://doi.org/10.1016/j.ijfoodmicro.2019.01.009.

Galli, V., Mazzoli, L., Luti, S., Venturi, M., Guerrini, S., Paoli, P., ... Pazzagli, L. (2018). Effect of selected strains of lactobacilli on the antioxidant and anti-inflammatory properties of sourdough. *International Journal of Food Microbiology, 286*, 55–65. https://doi.org/10.1016/j.ijfoodmicro.2018.07.018.

Galli, V., Venturi, M., Pini, N., Guerrini, S., Granchi, L., & Vincenzini, M. (2019). Liquid and firm sourdough fermentation: Microbial robustness and interactions during consecutive backsloppings. *LWT, 105*, 9–15. https://doi.org/10.1016/j.lwt.2019.02.004.

Gänzle, M. G., Loponen, J., & Gobbetti, M. (2008). Proteolysis in sourdough fermentations: Mechanisms and potential for improved bread quality. *Trends in Food Science & Technology, 19*, 513–521. https://doi.org/10.1016/j.tifs.2008.04.002.

Gänzle, M. G. (2014). Enzymatic and bacterial conversions during sourdough fermentation. *Food Microbiology, 37*, 2–10. https://doi.org/10.1016/j.fm.2013.04.007.

Giromini, C., Cheli, F., Rebucci, R., & Baldi, A. (2019). Dairy proteins and bioactive peptides: Modeling digestion and the intestinal barrier. *Journal of Dairy Science, 102*, 929–942. https://doi.org/10.3168/jds.2018-15163.

Gobbetti, M., De Angelis, M., Di Cagno, R., Calasso, M., Archetti, G., & Rizzello, C. G. (2018). Novel insights on the functional/nutritional features of the sourdough fermentation. *International Journal of Food Microbiology,* 30277. https://doi.org/10.1016/j.ijfoodmicro.2018.05.018 pii:S0168–1605(18).

Gupta, S., Ahsan, A. U., Wani, A., Khajuria, V., Nazir, L. A., Sharma, S., ... Ahmed, Z. (2018). The amino analogue of β-boswellic acid efficiently attenuates the release of pro-inflammatory mediators than its parent compound through the suppression of NF-κB/IκBα signalling axis. *Cytokine, 107*, 93–104. https://doi.org/10.1016/j.cyto.2017.12.004.

Gupta, S., Sharma, A. K., Shastri, V., Madhu, M. K., & Sharma, V. K. (2017). Prediction of anti-inflammatory proteins/peptides: An in-silico approach. *Journal of Translational Medicine, 15*, 7. https://doi.org/10.1186/s12967-016-1103-6.

Lugrin, J., Rosenblatt-Velin, N., Parapanov, R., & Liaudet, L. (2014). The role of oxidative stress during inflammatory processes. *Biological Chemistry, 395*, 203–230. https://doi.org/10.1515/hsz-2013-0241.

Milanovic, M., Kracht, M., & Schmitz, M. L. (2014). The cytokine-induced conformational switch of nuclear factor κB p65 is mediated by p65 phosphorylation. *The Biochemical Journal, 457*(3), 401–413. https://doi.org/10.1042/BJ20130780.

Minervini, F., Di Cagno, R., Lattanzi, A., De Angelis, M., Antonielli, L., Cardinali, G., ... Gobbetti, M. (2018). Lactic acid bacterium and yeast microbiotas of 19 sourdoughs used for traditional/typical italian breads: Interactions between ingredients and microbial species diversity. *Applied and Environmental Microbiology, 78*(4), 1251–1264. https://doi.org/10.1128/AEM.07721-11.

Pan, F., Wang, L., Cai, Z., Wang, Y., Wang, Y., Guo, J., ... Zhang, X. (2019). Soybean peptide QRPR activates autophagy and attenuates the inflammatory response in the RAW264.7 Cell Model. *Protein & Peptide Letters, 26*(4), 301–312. https://doi.org/10.2174/0929866526666190124150555.

Park, J. S., Park, M. Y., Cho, Y. J., Lee, J. H., Yoo, C. G., Lee, C. T., & Lee, S. M. (2016). Anti-inflammatory effect of erdosteine in lipopolysaccharide-stimulated RAW 264.7 Cells. *Inflammation, 39*(4), 1573–1581. https://doi.org/10.1007/s10753-016-0393-4.

Ramyaa, P., Krishnaswamy, R., & Padma, V. V. (2014). Quercetin modulates OTA-induced oxidative stress and redox signalling in HepG2 cells - up regulation of Nrf2 expression and down regulation of NF-κB and COX-2. *Biochimica et Biophysica Acta, Proteins and Proteomics, 1840*(1), 681–692. https://doi.org/10.1016/j.bbagen.2013.10.024.4.

Raveschot, C., Cudennec, B., Coutte, F., Flahaut, C., Fremont, M., Drider, D., & Dhulster, P. (2018). Production of bioactive peptides by Lactobacillus species: From gene to application. *Frontiers in Microbiology, 9*, 2354. https://doi.org/10.3389/fmicb.2018.02354.

Rizzello, C. G., Lorusso, A., Russo, V., Pinto, D., Marzani, B., & Gobbetti, M. (2017). Improving the antioxidant properties of quinoa flour through fermentation with selected autochthonous lactic acid bacteria. *International Journal of Food Microbiology, 241*, 252–326. https://doi.org/10.1016/j.ijfoodmicro.2016.10.035.

Secher, A., Kelstrup, C. D., Conde-Frieboes, K. W., Pyke, C., Raun, K., Wulff, B. S., & Olsen, J. V. (2016). Analytic framework for peptidomics applied to large-scale neuropeptide identification. *Nature Communications, 7*, 11436. https://doi.org/10.1038/ncomms11436.

Shadidi, F., & Li, Q. (2015). Biologically active peptides from foods. In Z. Ustunol (Ed.). *Applied Food Protein Chemistry* (pp. 75–98). Chichester: John Wiley & Sons Ltd.

Suetsuna, K., & Chen, J. R. (2002). Isolation and characterization of peptides with antioxidant activity derived from wheat gluten. *Food Science and Technology Research, 8*(3), 227–230. https://doi.org/10.3136/fstr.8.227.

Sun, Suling, Zhang, Hao, Shan, Kai, Sun, Tianjun, Lin, Mengyuan, Jia, Lingling, & Chen, Yong Q. (2019). Effect of different cereal peptides on the development of type 1 diabetes is associated with their anti-inflammatory ability: In vitro and in vivo studies. *Molecular Nutrition & Food Research, 63*(11), 1800987. https://doi.org/10.1002/mnfr.v63.1110.1002/mnfr.201800987.

Tolosa, L., Donato, M. T., & Gómez-Lechón, M. J. (2015). General cytotoxicity assessment by means of the MTT assay. *Methods in Molecular Biology, 1250*, 333–348. https://doi.org/10.1007/978-1-4939-2074-7_26.

Vaudel, M., Burkhart, J. M., Zahedi, R. P., Oveland, E., Berven, F. S., Sickmann, A., ... Barsnes, H. (2015). PeptideShaker enables reanalysis of MS-derived proteomics data sets. *Nature Biotechnology, 33*(1), 22–24. https://doi.org/10.1038/nbt.3109.

Xue, Q., Yan, Y., Zhang, R., & Xiong, H. (2018). Regulation of iNOS on immune cells and its role in diseases. *International Journal of Molecular Sciences, 19*(12), e3805. https://doi.org/10.3390/ijms19123805.

Zannini, E., Garofalo, C., Aquilanti, L., Santarelli, S., Silvestri, G., & Clementi, F. (2009). Microbiological and technological characterization of sourdoughs destined for bread-making with barley flour. *Food Microbiology, 26*, 744–753. https://doi.org/10.1016/j.fm.2009.07.014.

Zou, T. B., He, T. P., Li, H. B., Tang, H. W., & Xia, E. Q. (2016). The structure-activity relationship of the antioxidant peptides from natural proteins. *Molecules, 21*, 72. https://doi.org/10.3390/molecules21010072.