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RESEARCH ARTICLE

Lentils protein isolate as a fermenting substrate for the production of bioactive peptides by lactic acid bacteria and neglected yeast species

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Abstract

In the current trend where plant-based foods are preferred over animal-based foods, pulses represent an alternative source of protein but also of bioactive peptides (BPs). We investigated the pattern of protein hydrolysis during fermentation of red lentils protein isolate (RLPI) with various lactic acid bacteria and yeast strains. *Hanseniaspora uvarum* SY1 and *Fructilactobacillus sanfranciscensis* E10 were the most proteolytic microorganisms. *H. uvarum* SY1 led to the highest antiradical, angiotensin-converting enzyme-inhibitory and antifungal activities, as found in low molecular weight water soluble extracts (LMW-WSE). The 2039 peptide sequences identified by LMW-WSE were screened using BIOPEP UWM database, and 36 sequences matched with known BPs. Fermentation of RLPI by lactic acid bacteria and yeasts generated 12 peptides undetected in raw RLPI. Besides, *H. uvarum* SY1 led to the highest abundance (peak areas) of BPs, in particular with antioxidant and ACE-inhibitory activities. The amino acid sequences LVR and LVL, identified in the fermented RLPI, represent novel findings, as they were detected for the first time in substrates subjected to microbial fermentation. KVI, another BP highly characteristic of RLPI-SY1, was previously observed only in dried bonito. 44 novel potential BPs, worthy of further characterization, were correlated with antifungal activity.

INTRODUCTION

Dietary proteins represent essential macronutrients for human nutrition (Millward, 2023). Besides fulfilling a nutritional role, protein hydrolysis can lead to the release of bioactive peptides (BPs) with functional properties that include antihypertensive, antioxidant and immunomodulatory activities (Fadimu et al., 2022). Generally, BPs are specific protein fragments made up of short amino acids sequences (2 to 20 residues) of low-molecular-weight (<3 kDa). Their bioactivity and functionality are determined by the chain length, amino acid composition, sequence and molecular mass. For centuries and based on scientific evidence, milk and dairy products, particularly fermented dairy products, egg, meat and fish were praised as richest sources of such BPs (Chai et al., 2020).

The current state of public opinion, as well as the need to ensure a sustainable future supply, necessitate the shift from animal food to a higher percentage

of plant-based food from one side and encourage the scientific community to focus on the exploitation of plant matrices as alternative source of proteins for nutritional purposes but also to generate BPs. In recent decades, legumes (pulses) have been widely advocated as an alternative source of proteins in human nutrition. Lentil (*Lens culinaris* L.) is one of the most relevant cultivated pulse crops among the Leguminosae family, with global production of 5 million tons per year. The worldwide popularity and high consumption are related to the rich nutritional and functional profiles. The protein content ranges from 20 to 30%, comprising globulins (70%), albumins (16%), glutelins (11%) and prolamins (3%) (Boye et al., 2010). Several of these native proteins are known to be precursors of BPs with antioxidant activity, which may also act as inhibitors of angiotensin I converting enzyme (ACE) involved in rising blood pressure or have antifungal features (Ge et al., 2020). These characteristics, in combination with sustainable production and low costs, make red lentils an eligible source of alternative plant-based proteins to investigate.

BPs are inactive when encrypted within intact protein of lentils and only become activated when cleaved by enzymatic and chemical hydrolysis during food processing, as well as via gastrointestinal digestion or by the proteolytic capabilities of microorganisms at gut level (Ashaolu, 2020; Korhonen & Pihlanto, 2006). When considering food processing, chemical hydrolysis by acid (HCl) has been linked to health issues caused by the production of carcinogen compounds (Ulug et al., 2021). While enzymatic approach represents the safe and most preferred method for degrading food proteins and releasing smaller peptide fragments and amino acids (Zambrowicz et al., 2013). Interestingly, enzymatic hydrolysis to obtain BPs can be catalysed by proteases, which are found in a variety of microorganisms, or can be released during the microbial fermentation (Mamo & Assefa, 2018). According to the available literature, production of BPs from protein plant primarily soybeans, rice beans, corn and lentils was optimized through fermentation using *Bacillus* spp. or filamentous fungi (Padhi et al., 2022). To the best of our knowledge, few studies on the release of BPs by lactic acid bacteria (LAB) have been addressed to such plant substrates, although proteolytic activity and potential application for enhancing the functional features of legumes have been demonstrated. Only a small percentage of the wide range of LAB have been previously associated with a particular proteolytic activity or the hydrolysis of protein into BPs (Raveschot et al., 2018). This was also confirmed on red lentils used as substrate. Indeed, lactic acid fermentation of red lentils using *Lactiplantibacillus plantarum* increased protein digestibility, degradation of antinutritional compounds and reduction of trypsin inhibitory activity (De Pasquale et al., 2020). The same properties were highlighted during red lentils-based sourdough fermentation but using *Fructilactobacillus sanfranciscensis* as starter (Montemurro et al., 2019). Yeast proteolytic activity, on the other hand, has never been investigated for these aims in such substrates, notwithstanding the fact that among the yeasts under investigation, *Kazachstania unispora* played a prominent role in the release of several BPs during sourdough fermentation (Korcari et al., 2021).

In the last few years, the use of lentil protein isolates in food applications has become widespread, as the extraction process by appropriate processing techniques is an efficient strategy to reduces the level of antinutritional compounds naturally present in lentils, and increases the digestibility of proteins, due to the lower degree of interactions and complexations among molecules (Joehnke et al., 2021). Although several advantages of using protein isolates are known, their exploitation as source of BPs has so far been neglected. Both the degree of denaturation, which may result from the isolation process, and the profile of the isolated proteins may affect the release of BPs and their functionality. Only a few recent studies have highlighted the potential of lentils as a substrate for release of BPs by enzymatic hydrolysis (Mohammadi et al., 2022). Consequently, we proposed to investigate the hydrolysis pattern during fermentation of red lentils protein isolate (RLPI) with various LAB and yeast strains, some of which belong to species whose role in BPs release has often been overlooked. To this end, Ultra High-Performance Liquid Chromatography-High-Resolution Tandem Mass Spectrometry (UHPLC-HRMS2) and bioinformatic tools were employed to identify and characterize BPs in fermented RLPI. Then, we associated the release of BPs by biological hydrolysis with anti-ACE, antioxidant and antifungal properties of fermented RLPI.

EXPERIMENTAL PROCEDURE

Raw materials

Red lentils protein isolate was obtained by aqueous extraction of native lentil flour and provided from Fraunhofer IVV (Freising, Germany) as one of the selected alternative protein sources for Smart Protein Project (EU Framework Program for Research and Innovation – Horizon 2020; https://smartproteinproject.eu), with proximate composition indicated in Table S1. The protein isolate was divided into aliquots and stored under vacuum conditions at room temperature until further use. In order to carry out a preliminary microbiological characterization of the isolate, bacteria and yeasts were isolated and identified by partial sequencing of

the 16S rRNA and 26S rRNA genes as described by Lhomme et al. (2015).

Microorganisms and culture conditions

Three strains of LAB and two of yeasts, belonging to the Culture Collection of the Department of Soil, Plant and Food Science, University of Bari Aldo Moro (Bari, Italy) and the Micro4Food of Faculty of Agriculture, Environmental, and Food Sciences, Libera Università di Bolzano (Bolzano, Italy) were used as starters for RLPI fermentation. All strains were previously isolated from plant-based products (Table S2). Cultures were maintained as stocks in 20% (v v^{-1}) glycerol at −20°C and routinely propagated at 30°C for 24 h in MRS broth for LAB and SDA broth for yeasts.

RLPI fermentation

Red lentils protein isolate (50 g) was mixed with water (100 g) to create a fermentable dough of dough yield (DY = [dough weight/flour weight]*100) of 300. The resulting dough, supplemented with glucose (1%, w w^{-1}), was singly fermented by pure culture of LAB and yeast strains. Cells were harvested after 24 h by centrifugation (11,000 ***g***, 10 min at 4°C), washed twice in 50 mM sterile potassium phosphate buffer (pH 7.0) and singly inoculated in RLPI dough to a final cell density of ca. 7.0 and 5.0 Log CFU mL^{-1} for LAB and yeasts, respectively. RLPI was fermented at 30°C for 8 days. Fermentation conditions were defined on the basis of technical and scientific considerations supported by the literature (Acín Albiac et al., 2020; Di Cagno et al., 2020; Filannino et al., 2016). RLPI without bacterial inoculum and incubation (RLPI-Raw) and RLPI without inoculum but incubated at 30°C (RLPI-Unstarted) were used as the controls. Samples were taken at the beginning of the fermentation (D0), after 2 (D2), 4 (D4), 6 (D6) and 8 (D8) days of fermentation. Sampling timepoints were chosen to ensure wide coverage of fermentation processes, which varied in duration. All samples were subjected to the freeze-drying treatment (Lyostar II, FTS kinetics, Stone Ridge, NY, USA).

Microbial growth and acidification

Samples of RLPI (10 g) were suspended into sterile 0.9% (w v^{-1}) NaCl and homogenized using a Stomacher apparatus (Seward, London, UK) for 3 min at room temperature. Serially diluted aliquots were plated on different agar media. Mesophilic lactic acid bacteria and yeasts were determined on MRS agar (Oxoid) containing 0.1% of cycloheximide (Sigma Chemical Co.) at 30°C for 48 and 72 h under anaerobiosis and on SDA (SDA, Oxoid), added of 150 ppm chloramphenicol at 30°C for 72 h, respectively. The value of pH was measured by a Foodtrode electrode (Hamilton, Bonaduz, Switzerland).

Sugars and organic acids quantification

Water-soluble extract (WSE) of raw-, unstarted and started RLPI was obtained by mixing 2 g of freeze-dried RLPI powder with 18 mL of HCl (0.1 mM), incubated at 4°C for 1 h under stirring conditions (200 rpm) and centrifuged (12,000 × ***g*** for 10 min). Water soluble extracts (WSE) were filtered (0.22 μm membrane filter, Millipore Corporation) and stored at −20°C until further use. HPLC analysis using a Spherisorb column (Waters, Millford, USA) and a Perkin Elmer 200a refractive index detector was applied to determine the concentration of glucose (Tlais et al., 2022). Lactic and acetic acids were determined by HPLC analysis equipped with an Aminex HPX-87H column (ion exclusion, Biorad) and a UV detector operating at 210 nm (Tlais et al., 2022). Organic acids and glucose standards were purchased from Sigma-Aldrich (Steinheim, Germany).

Total protein quantification

Total protein concentration was determined in the water-soluble extracts (WSE) of all the samples using Bradford assay. In 96-well microplate reader (Tecan Infinite 200, Italy), the absorbance of supernatants was measured at 590 nm and protein concentration was calculated using the calibration curve of bovine serum albumin as standard.

Peptides profiling and quantification

In order to separate the active peptide's fraction, the WSE was further subjected to ultrafiltration (molecular weight cut-off <3 kDa) as previously described by Tagliazucchi et al. (2017), with some modifications. For this purpose, 2 mL of the sample was placed in a Vivaspin®20 column, 3000 MWCO-PES (Sartorius, Italy), and subjected to centrifugation at 4000 ***g*** for 40 min. The obtained low molecular weight water soluble extract (LMW-WSE) was used for next investigations.

The peptide concentration was determined by the TNBS (2,4,6-trinitrobenzenesulfonic acid) method as originally described by Adler-Nissen (1979). The results were expressed as mg leucine (Leu) equivalent g^{-1} of RLPI.

Peptides profiles in LMW-WSE were determined by reversed-phase fast performance liquid chromatography

(RP-FPLC), using a Resource RPC column and 'AKTA FPLC equipment', with the UV detector operating at 214 nm (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) as previously described by Pontonio et al. (2020). Briefly, 1.5 mL of LMW-WSE aliquots containing peptides were loaded onto the column. Gradient elution was performed at a flow rate of 1 mL min^{-1} using a mobile phase composed of water and acetonitrile (CH_3CN), containing 0.05% trifluoroacetic acid. The concentration of CH_3CN was increased linearly from 5 to 46% between 16 and 62 min and from 46 to 100% between 62 and 72 min.

In vitro antifungal activity

Hyphal radial growth rate assay was used to determine the in vitro antifungal activity of the LMW-WSE, as previously described by Rizzello et al. (2011) with some modifications. Briefly, LMW-WSE was added (25%, v v^{-1} final concentration) to Potato Dextrose Agar (PDA) medium (Oxoid Ltd, Basingstoke, Hampshire, England) and poured into mini-Petri dishes. Control plates contained PDA alone. A 5 mm Ø of fresh mycelia of *Penicillium roqueforti* P1 was placed as mould indicator in the centre of the mini-Plates containing the culture medium. The hyphal radial growth rate was measured after 5 days of incubation at 25°C and the related inhibition percentage was calculated as follows:

$$\text{Growth inhibition}\,(\%) = \left[(\text{mycelial growth under control conditions} - \text{mycelial growth in the presence of LMW} - \text{WSE})/\text{mycelial growth under control conditions}\right] \times 100.$$

ACE-inhibitory activity

ACE-inhibitory activity was measured in vitro by the spectrophotometric assay using the tripeptide, N-[3-(2-furyl)acryloyl]-L-phenylalanyl-glycyl-glycine (FAPGG) as (Ronca-Testoni, 1983). The reaction was monitored at 340 nm for 10 min. In order to calculate IC_{50} values, ACE-inhibitory activity assay was carried out using different concentrations of the LMW-WSE (<3 kDa). IC_{50} was defined as the amount of LMW-WSE, expressed in μL, required to inhibit 50% of the enzymatic activity. The IC_{50} values were determined using nonlinear regression analysis and fitting the data with the log (inhibitor) versus the response model generated by GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA).

ABTS + radical scavenging activity

ABTS radical scavenging activity of LMW-WSE was estimated by Antioxidant Assay kit (Sigma-Aldrich) according to the manufacturer's instructions. The principle of ABTS assay is formation of a ferryl myoglobin radical from metmyoglobin and hydrogen peroxide, which oxidizes the ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) to produce a radical cation, $ABTS^+$, a soluble chromogen that is green in colour and can be determined spectrophotometrically at 405 nm. Trolox, a water-soluble vitamin E analogue, was used as a control antioxidant.

Identification of low molecular weight peptides by UHPLC/HR-MS2

Low molecular weight peptides in LMW-WSE were identified by UHPLC/HR-MS2 (UHPLC Ultimate 3000; Thermo Scientific, San Jose, CA, USA; Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer, Thermo Scientific, San Jose, CA, USA) equipped with a C18 column (Zorbax SB-C18 Reversed-phase, 2.1 × 50 mm, 1.8 μm particle size, Agilent Technologies, Santa Clara, CA, USA), as previously described by Martini et al. (2020). The MS data was first transformed in a .mgf file and then processed with the MASCOT software (Matrix Science, Boston, MA, USA) for peptide sequencing and identification. The parameters used for the identification process were: enzyme, none; peptide mass tolerance, ±5 ppm; fragment mass tolerance, ±0.1 Da; variable modification, Deamidation (NQ), oxidation (M) and phosphorylation (ST); the maximal number of post-translational modifications permitted in a single peptide, 1. Only peptides identified with a significance threshold of $p < 0.05$ were considered in the following analysis.

Label-free MS peak relative quantification and data filtering

The creation of a library of peptides and their fragments, labelling, relative quantification and data filtering of the identified peptides was performed with Skyline 22.2 software as reported by Dallas and Nielsen (2018). Peaks that did not meet the criteria or were too close to the noise level to be visually discernible were excluded from the data set. The criteria used were a mass error between 5 and −5 ppm and an idotp (isotope scalar product) score of ≥0.8. Through the systematic manual screening of the exported data, peptides whose origin was not from legumes proteins were also excluded. Furthermore, isotopic labelled peptides and identical modified peptides (i.e. different protonation pattern, presence of modifications like oxidation and deamidation) were grouped and the peak area was summed. Peptides identified in the samples were examined for homology with bioactive peptides identified in the literature by using the Bioactive Peptide Database BIOPEP UWM (Minkiewicz et al., 2019).

Statistical analysis

All analyses were carried out in triplicate on three biological replicates. Data were subjected to analysis of variance by the General Linear Model (GLM) of R statistical package. Pairwise comparison of treatment means was achieved by Tukey-adjusted comparison procedure with *p*-value (p) < 0.05. The relative quantification data of bioactive peptides were subjected to Kruskal–Wallis's test. Multiple pairwise comparisons were done using Dunn's procedure with a *p*-value ($p < 0.05$). Spearman's rank correlation matrix and *p*-values were generated by cor.test and visualized by corrplot package.

RESULTS AND DISCUSSION

Red lentils protein isolate (RLPI) fermentation

We used a RLPI with a protein content of 807.10 ± 20.10 mg g^{-1} DW and a peptide content of 55.35 ± 0.37 mg Leu eq. g^{-1} DW as substrate for fermentation (Table S1). *Lactiplantibacillus plantarum* LM1.3, *Lacticaseibacillus rhamnosus* ATCC53103, and *Fructilactobacillus sanfranciscensis* E10 as well as *Hanseniaspora uvarum* SY1 and *Kazachstania unispora* KFBY1 (Table S2) were chosen as single starter based on their pro-technological and proteolytic properties assayed in preliminary trials (data not shown) and based on the available literature (De Pasquale et al., 2020). Due to the low sugar content and to promote microbial growth and activity, resultant dough was supplemented with 1% (w w^{-1}) of glucose before fermenting with LAB and yeast starters. Proteolysis is fermentation-time dependent and longer fermentation maximizes BPs release (Wei et al., 2021). Consequently, we monitored RLPI dough fermentation at 30°C for 8 days. Doughs sampled every 2 days were analysed to ensure wide coverage of changes during the fermentation processes. RLPI-Unstarted was included as a control to account for the autochthonous microbiota of RLPI, which had a heterogeneous composition (Figure S1). As estimated on Man, Rogosa and Sharpe media (MRS) and Sabouraud Dextrose Agar (SDA) agar, the cell density of presumptive LAB and yeasts in raw RLPI dough were 4.25 ± 0.43 and 4.01 ± 0.27 Log CFU g^{-1}, respectively. The cell density of LAB in RLPI-unstarted increased suddenly during the first 2 days of fermentation (7.03 ± 0.36 Log CFU g^{-1}), and then continued to increase until the end of fermentation reaching 9.02 ± 0.19 Log CFU g^{-1}. The yeast count followed almost the same trend (Figure 1a). As expected, started fermentation ensured larger number of

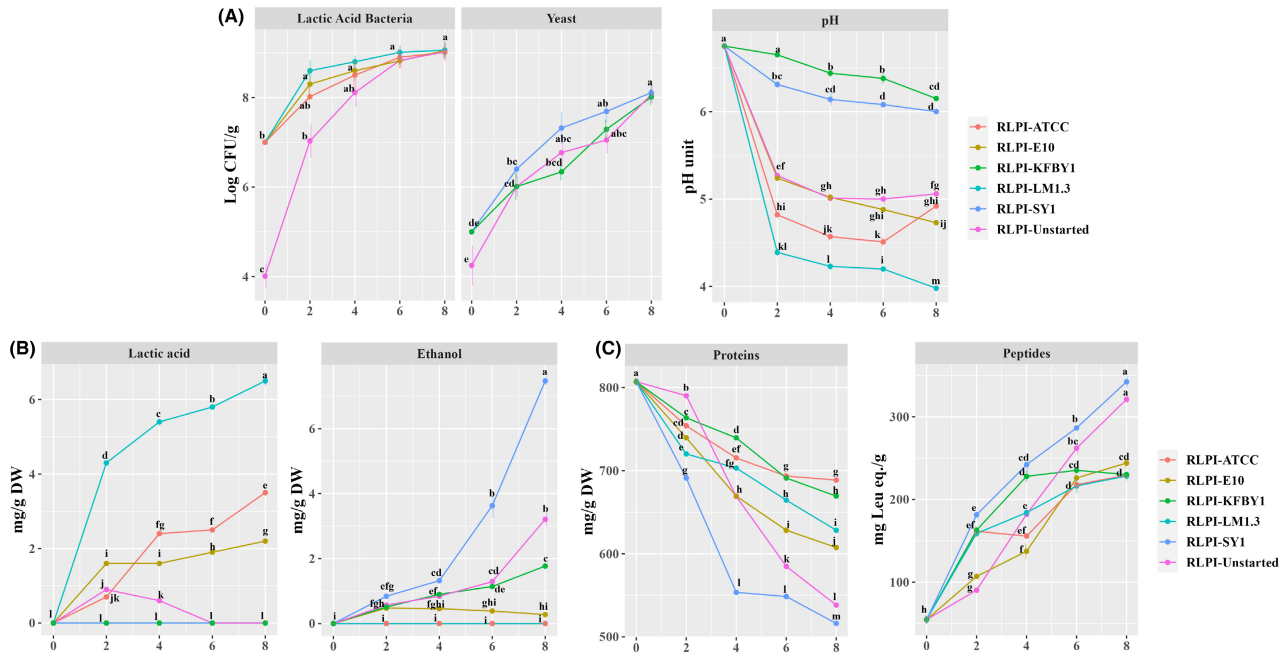

FIGURE 1 Cell density of lactic acid bacteria and yeasts (Log CFU g^{-1}) and pH variation (panel A), lactic acid (mg g^{-1} DW) and ethanol (mg g^{-1} DW) production (B), protein hydrolysis (mg g^{-1} DW) and peptide release (mg Leu eq. g^{-1}) (C) during the fermentation of red lentil protein isolate (RLPI) carried out at 30°C for 8 days. RLPI was fermented with *Lactiplantibacillus plantarum* LM1.3 (RLPI-LM1.3), *Lacticaseibacillus rhamnosus* ATCC53103 (RLPI-ATCC), *Fructilactibacillus sanfranciscensis* E10 (RLPI-E10), *Kazachstania unispora* KFBY1 (RLPI- KFBY1) and *Hanseniaspora uvarum* SY1 (RLPI- SY1). RLPI without microbial inoculum and incubated under the same conditions (RLPI-Unstarted) was used as the control. Results are shown as the means (±SD) of three biological replicates analysed in triplicate. Data points with different superscript letters (a–m) differ significantly ($p < 0.05$).

LAB and yeasts among the different species throughout the fermentation, which reached its highest values after only 4 days of fermentation (9.06 ± 0.16 and 8.11 ± 0.15 Log CFU g^{-1}, respectively), and then remained almost constant until the end of the fermentation (Figure 1a). The variations in LAB cell density mirrored the changes in pH. RLPI had initial pH value of 6.75 ± 0.03, which sharply dropped after 2 days of fermentation in RLPI-unstarted and RLPI fermented with LAB starters, albeit to varying degrees, and then remained almost stable with few fluctuations until the end of the fermentation. RLPI fermented with *Lp. plantarum* showed the highest reduction in pH (3.98 ± 0.02) after 8 days of fermentation. On the other hand, RLPI fermented with yeasts showed only a slight decrease in pH over time. At the end of the fermentation, it was 6.15 ± 0.02 for RLPI-SY1 and 6.08 ± 0.03 for RLPI-KFBY1 (Figure 1a).

A complete consumption of supplemented glucose (10.3 mg g^{-1} DW) was found after only 2 days in all fermented samples. In RLPI started with LAB, the main microbial metabolite was lactic acid (Figure 1b). The highest concentration was quantified in RLPI fermented with *Lp. plantarum* LM1.3 (6.50 ± 0.12 mg g^{-1} DW), followed by *Lc. rhamnosus* ATCC53103 (3.50 ± 0.06 mg g^{-1} DW) and *F. fructosus* E10 (2.20 ± 0.02 mg g^{-1} DW). Ethanol (0.27 ± 0.06–0.48 ± 0.09 mg g^{-1} DW) and acetic acid (0.94 ± 0.11–1.41 ± 0.18 mg g^{-1} DW) were also found in RLPI-E10. On the other hand, the main metabolite in the yeast-fermented samples was ethanol, with the highest amount produced by *H. uvarum* SY1 during the last 2 days (7.47 ± 0.12 mg g^{-1} DW), followed by *K. unispora* KFBY1 (1.77 ± 0.09 mg g^{-1} DW) (Figure 1b). Under spontaneous fermentation conditions, lactic acid concentration increased up to 2 days of fermentation (0.90 ± 0.07 mg g^{-1} DW), and then had a downward trend, reaching zero value after 6 days of fermentation (Figure 1b). Acetic acid and ethanol were also found during the last 2 days of fermentation of RLPI-Unstarted (1.72 ± 0.32 mg g^{-1} DW and 4.28 ± 0.28 mg g^{-1} DW, respectively) (Figure 1b).

Protein and peptides

Protein hydrolysis and peptides release were monitored throughout the fermentation by using WSE and LMW-WSE, respectively. According to our observations, RLPI-Raw dough had an initial protein content of 807.1 ± 20.1 mg g^{-1} DW and peptides of 54.8 ± 4.2 mg g^{-1} DW. Overall, all microorganisms in fermented and unfermented RLPI were able to hydrolyze proteins into peptides in rising trend although with varying magnitude over 8 days of fermentation. Protein content was mostly reduced in RLPI-SY1 (36%), followed by RLPI-Unstarted (33%), RLPI-E10 (25%) and RLPI-LM1.3 (22%), with the least reduction in RLPI-KFBY1 (17%) and RLPI-ATCC (14%). As expected, the reduction in proteins was associated with the high release of peptides. Indeed, the highest level of peptide concentration was attributed to RLPI-SY1 (342.3 ± 6.45 mg Leu eq. g^{-1} DW) and RLPI-Unstarted (321.1 ± 4.09 45 mg Leu eq. g^{-1} DW) after 8 days of fermentation. In the other fermented samples, the level of peptides peaked after 6 days (228.3 ± 5.16 to 244.04 ± 6.21 45 mg Leu eq. g^{-1} DW) and remained constant until the end of the fermentation (Figure 1c). In all fermented samples, protein hydrolysis was more pronounced in the latter part of the incubation, in concomitance with the depletion of fermentable sugars. This is consistent with the need for microorganisms to obtain alternative energetic sources via extracellular proteases and peptidases, which hydrolyse protein into free amino acids and peptides (Diether & Willing, 2019). The diverse pools of proteolytic enzymes that are unique to each microorganism are linked to the variable degrees of protein hydrolysis, supporting our findings (Elhalis et al., 2021). Different patterns of protein hydrolysis mirrored distinct levels of peptides formation, distinguishing *H. uvarum* SY1 as the most powerful proteolytic starter. This finding was consistent with previous studies, in which *H. uvarum* attained the best metabolic and proteolytic activity performance among non-*Saccharomyces* species under winemaking process (Ciani & Comitini, 2019). *Bacillus* spp. is the predominant endogenous microbiota in legumes flours. Its crucial role to release BPs with high antioxidant activity was shown when used as starter to ferment corn, rice and even lentils (Sabater et al., 2020). Among LAB starters, the proteolytic activity was species-dependent, with *F. sanfranciscensis* E10 being the most effective. Compared with other LAB species, it is generally considered to be weakly proteolytic, as it relies on a complex proteolytic system to release amino acids and peptides from the protein-rich substrates (Savoy De Giori & Hébert, 2001).

Peptides profiling by RP-FPLC

Aiming to further investigate the protein hydrolysis, peptide profiles of raw and fermented RLPI were analysed through RP-FPLC. Significant differences in the peptide profiles were found during fermentation and among the samples (Figure 2). Chromatograms revealed the higher number and intensity of peptide peaks in unstarted (RLPI-Unstarted), SY1-fermented (RLPI-SY1) and E10-fermented (RLPI-E10) RLPI than in raw (RLPI-Raw) and to lesser extent in RLPI fermented by KFBY1 (RLPI-KFBY1) and ATCC53103 (RLPI-ATCC) (Figure 2). Besides, the time of fermentation affected the profile of peptide, with most

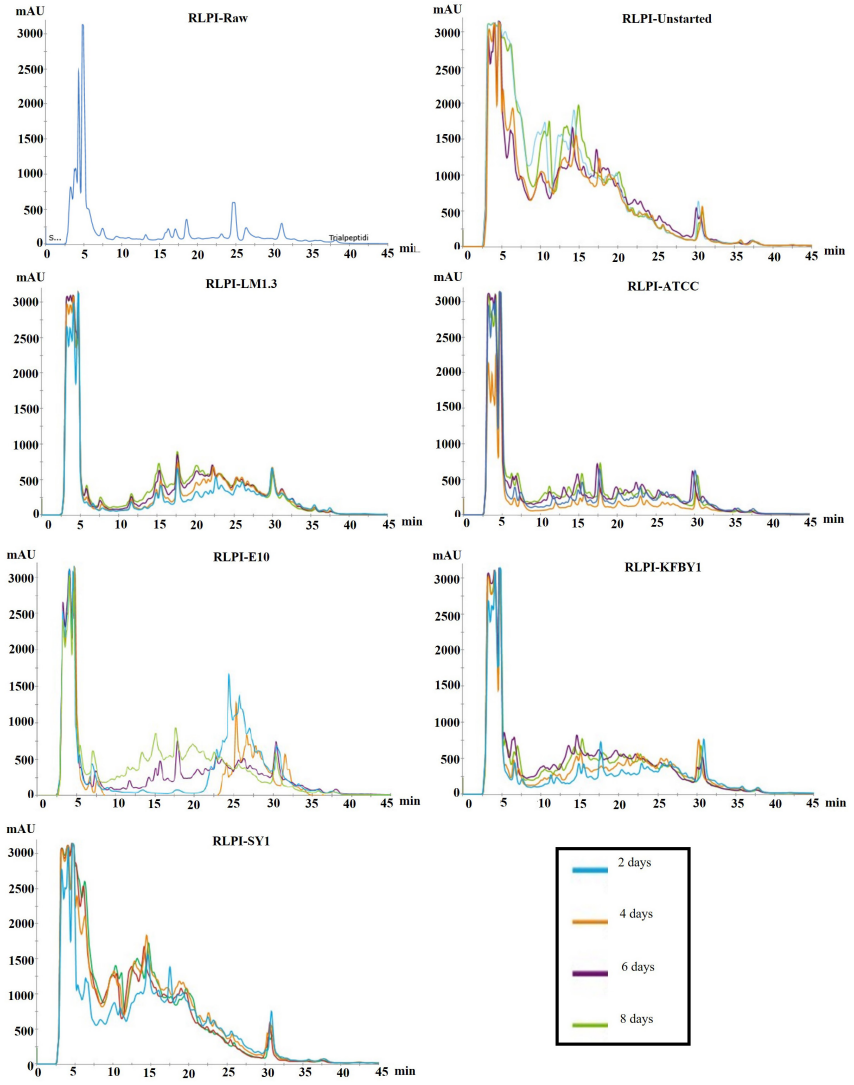

FIGURE 2 Peptides profile obtained through RP-FPLC (detector 240 mm) chromatograms during the fermentation of red lentil protein isolate (RLPI) carried out at 30°C for 8 days. RLPI was fermented with *Lactiplantibacillus plantarum* LM1.3 (RLPI-LM1.3), *Lacticaseibacillus rhamnosus* ATCC53103 (RLPI-ATCC), *Fructilactibacillus sanfranciscensis* E10 (RLPI-E10), *Kazachstania unispora* KFBY1 (RLPI-KFBY1) and *Hanseniaspora uvarum* SY1 (RLPI-SY1). RLPI without microbial inoculum and incubated under the same conditions (RLPI-Unstarted) was used as the control. In blue, the chromatograms corresponding to time = 2 days; in orange time = 4 days; in purple time = 6 days; in green the chromatograms corresponding to time = 8 days of fermentation. 'Raw' reports the chromatogram of the raw unfermented red lentils protein isolate at time $t = 0$.

peptides occurring mainly after 8 days of fermentation. RP-FPLC, as partial informative tool, confirmed the species-specific features and the shift of the peaks occurring throughout fermentation, highlighting the high variability in the formation of potential BPs (Pontonio et al., 2020), but leaved a gap about the identity of peptides and their functional properties. Only the fermented samples (after 8 days) were considered for further analysis.

Antifungal activity

The inhibitory effect of raw and fermented RLPI against mould indicator *P. roqueforti* P1 was determined by using peptide extracts (LMW-WSPE). *P. roqueforti* is considered as one of the principal moulds causing bread spoilage worldwide (Bernardi et al., 2019). All LMW-WSE from fermented samples markedly ($p < 0.05$) increased the inhibition of radial hyphal growth rate of *P. roqueforti* P1 when compared to the RLPI-Raw (12.5 ± 1.52%). In particular, RLPI-SY1 showed the highest inhibitory actions (c.a. 60.4 ± 0.7% of inhibition) together with RLPI-Unstarted (60.1 ± 1.06%). A moderate activity was detected with RLPI-LM1.3 (56 ± 1.06%) and RLPI-KFBY1 (54.2 ± 0.78%). The lowest radial growth inhibition compared to the control was observed in RLPI-ATCC (50 ± 0.85%) and RLPI-E10 (35.4 ± 1.13%) (Figure 3a, Figure S2). The contribution of LAB, notably *Lp. plantarum* and *Lc. rhamnosus* under proteolytic actions, in generating antifungal BPs against several *Penicillium* spp. was confirmed by several studies (Ramos-Pereira et al., 2021). On the other hand, to the best of our knowledge, there is no available data in the literature on the potential of *H. uvarum*, which inhibited *P. roqueforti* P1 the most, on the release of antifungal peptides. With regard to RLPI-Unstarted, it is crucial to consider the inherent variability and heterogeneity of the autochthonous RLPI microbiota (Figure S1). The variability significantly diminishes the utility for industrial applications of any positive results obtained during spontaneous fermentation. Indeed, the seasonal and geographical variability, coupled with the influence of the protein isolation process, can introduce microbial

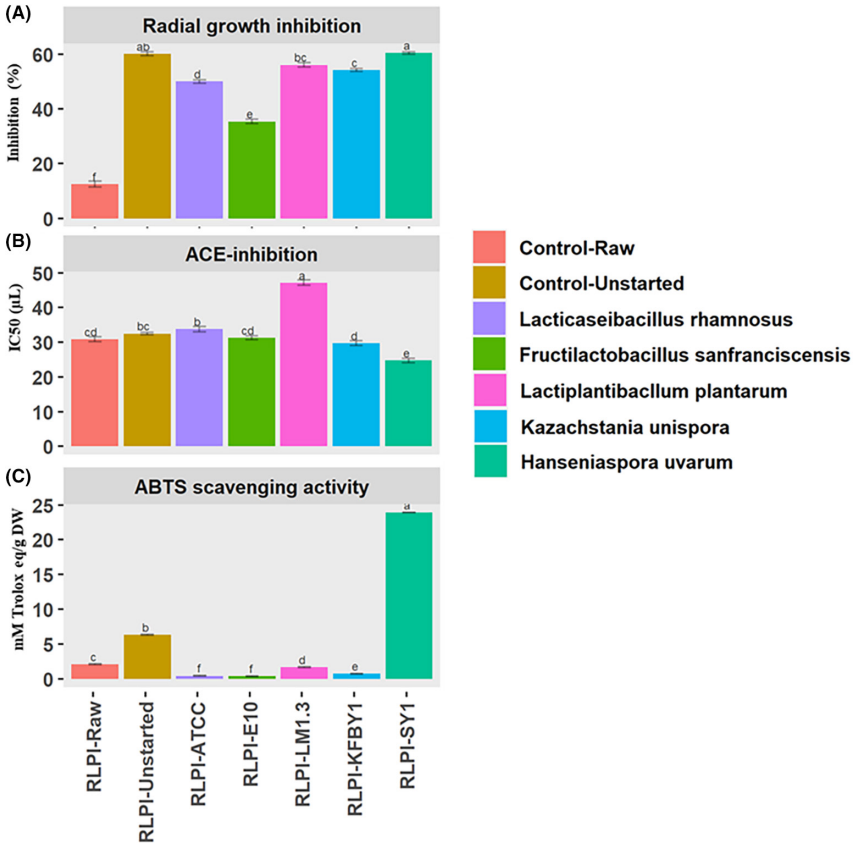

FIGURE 3 In vitro determination of radial growth inhibition against *Penicillium roqueforti* P1 (panel A), ACE-inhibitory (B) and ABTS scavenging activity (C) of low molecular weight water soluble extracts (LMW-WSE) obtained from raw red lentils protein isolate (RLPI-Raw), RLPI without microbial inoculum (RLPI-Unstarted) and Fermented-RLPI, which were incubated at 30°C for 8 days. Fermentation was done spontaneously (RLPI-Unstarted) or with *Lactiplantibacillus plantarum* LM1.3 (RLPI-LM1.3), *Lacticaseibacillus rhamnosus* ATCC53103 (RLPI-ATCC), *Fructilactibacillus sanfranciscensis* E10 (RLPI-E10), *Kazachstania unispora* KFBY1 (RLPI- KFBY1) and *Hanseniaspora uvarum* SY1 (RLPI- SY1). Results are shown as the means (±SD) of three biological replicates analysed in triplicate. Bars with different superscript letters (a–f) differ significantly ($p < 0.05$).

diversity resulting in low product standardization and non-reproducibility of the fermentation process.

ACE-inhibitory activity of LMW-WSPE

The renin-angiotensin system (RAS), a proteolytic system largely involved in the regulation of fluid balance and blood pressure, is one of the most significant metabolic processes involved in the management of cardiovascular homeostasis. Angiotensinogen, a protein primarily generated in the liver, is transformed to angiotensin I (Ang I) by the action of renin, an enzyme primarily secreted by the kidney. ACE, which is typically generated in the lungs, converts Ang I into angiotensin II (Ang II) (Marques et al., 2012). The inhibitory effect towards ACE could significantly impact human health. The in vitro ACE-inhibitory activity assay was performed to assess the antihypertensive potential of the raw and fermented RLPI, and the results were expressed as IC_{50}, which defines the quantity of LMW-WSPE extract needed to inhibit 50% of the ACE activity. Red lentil protein isolate fermented with *H. uvarum* SY1 (RLPI-SY1) was the only sample that showed significant ($p < 0.05$) ACE inhibition with the lowest IC_{50} (24.75 ± 0.77 µL) compared with RLPI-Raw (30.79 ± 0.87 µL) (Figure 3b). Compared with the RLPI-Raw, RLPI-Unstarted, RLPI-E10 and RLPI-KFBY1 showed almost same IC_{50} values with no statistical significance ($p > 0.05$). The negative effect of started fermentation on ACE inhibition was significantly found ($p < 0.05$) in RLPI-LM1.3 (47.13 ± 0.97 µL) and RLPI-ATCC (33.76 ± 0.92 µL) (Figure 3b). To date, researchers have studied the possible inhibitory effect of red lentil protein hydrolysates. The generation of peptides with this bioactivity was found in RLPI hydrolysed with commercial trypsin (Boye et al., 2010). Although the proteolytic system of various lactic acid bacteria has been effectively exploited through fermentation to create BPs with such activity, none of the LAB starters used in our study were able to release anti-ACE peptides. Conversely, our findings demonstrated the efficiency of *H. uvarum* SY1 in suppressing ACE. Even yeast cells have long been explored as a source of BPs with ACE-inhibitory (Mirzaei et al., 2021), their use as starters to drive

protein hydrolysis and the release of anti-ACE peptides has not been reported previously.

ABTS scavenging activity

Antioxidant peptides can be used to prevent oxidative stress and food quality deterioration, making them an appealing strategy for reducing economic losses in food production while simultaneously improving public health (Chakrabarti et al., 2014). Thus, the ABTS radical scavenging capacity of LMW-WSE from raw and fermented RLPI was also evaluated. Compared with RLPI-Raw (2.12 ± 0.06 mM Trolox eq. g^{-1} DW), a remarkable increase ($p < 0.05$) of the ABTS radical scavenging capacity was found in LMW-WPE of RLPI-SY1 (23.92 ± 0.01 mM Trolox eq. g^{-1} DW) and RLPI-Unstarted (6.36 ± 0.08 mM Trolox eq. g^{-1} DW). The thermal stability of the ABTS radical scavenging capacity of LMW-WSE from RLPI-SY1 was confirmed after heat treatment (100°C for 5 min). Unexpectedly, the antioxidant activity of RLPI was negatively affected by the lactic acid fermentation (Figure 3c). This contradicted previous finding focused on the potential role of *Lp. plantarum* strains in whole cow's milk in the formation of antioxidant-rich hydrolysate (Aguilar-Toalá et al., 2017), and *Lc. rhamnosus* in releasing antioxidant peptides (Solieri et al., 2015).

Peptidomic profiles of unfermented and fermented RLPI

Aiming to evaluate the effects of fermentations on peptide release, their profile was analysed through HPLC-HRMS. In recent years, HRMS-based peptidomics analysis has emerged as a robust and sensitive method for comprehensively mapping the peptidome present in various matrices. MS-based peptidomics analysis is therefore well-suited for monitoring the abundances of already known peptides in samples, although its application in discovering novel BPs shows particular challenges due to the large background of detected degradation products and inactive precursors (Aydoğan, 2020). The analysis revealed a total of 2039 distinct peptides among all samples, with only 391 peptides found in the RLPI-Raw. The consequence of different proteolytic activity related to various starters on the quantity and variety of peptides was remarkable (Figure 4a,b). The use of LAB caused the greatest rise in the number of peptides, in which RLPI-ATCC (1520 peptides) and RLPI-E10 (1506) exhibited the most peptide diversity among all the samples. Considering yeast fermentations, RLPI-SY1 showed the highest diversity of peptide substrate after RLPI-ATCC and RLPI-E10, whereas RLPI-KFBY1 demonstrated the lowest diversity. The effect of spontaneous fermentation on the proteolytic activity can be observed in RLPI-Unstarted, which showed a high number of distinct peptides (1421) (Figure 4a). The jitter plot in Figure 4b offers a precise depiction of the molecular weight distribution of identified peptides. This representation was feasible through the implementation of HRMS/MS, which ensures enhanced accuracy and resolution compared to traditional techniques, particularly for peptides smaller than 3 kDa (Hellinger et al., 2023; Rivera et al., 2018).

BPs identification and relative quantification

Peptides identified in analysed samples were compared for sequence homology with known BPs by using BIOPEP UWM database. The database, which has over 4600 BPs sequences, has recently gained popularity in food and nutrition science as a source of data about these molecules, which are of particular interest as potential ingredients of functional foods and nutraceutical applications (Minkiewicz et al., 2019). Most of the peptides in raw and fermented RLPI were related to parental proteins from chickpea and a few other legumes. From the database search, 36 out of 2039 peptide sequences had 100% identity with previously recognized and validated BPs sequences (Figure 5a,b). First, the bulk of the discovered BPs were linked to plant proteins. In fact, the majority of BPs research is mainly focused on precursor proteins from dairy, meat and fish, with a minor portion on plant-based proteins (Bhat et al., 2015). Among the 36 BPs, 73% were ACE-inhibitory, 22% antioxidant, and 5% featured for other bioactivities (i.e. DPPIV-inhibitory and antidiabetic). 13 of these BPs (ALEPDHR, FAP, FFI, KLP, LLP, LLPH, LNF, LVR, PLLR, PPP, TETWNPNHPEL, VVR, YLR), prevalently featuring ACE-inhibitory and antioxidant activity, were founded exclusively in the fermented RLPI at different amounts. Regardless of peaks intensity, the highest number of BPs were found in RLPI-SY1 (36) and RLPI-Unstarted (35), whereas the remaining samples showed lower numbers ranging between 27 and 32 (Figure 5b). Moreover, only 12 bioactive peptides were shared among all the samples with RLPI-Raw counting the lowest variety (Figure 5b,c). The effect of fermentation on the BPs resulted evident from the relative quantification analysis (Table S3, Figure 5b,c). The identified BPs originated from proteins that consistently exist in lentils and are minimally affected by agronomic and environmental factors (Table S3). With a few exceptions, each BP showed a considerable increase in peptide abundance from the RLPI-Raw. In particular, the RLPI-SY1 resulted in the highest accumulative intensity of BPs, followed by RLPI-Unstarted. Despite the high number of BPs found in LAB-fermented samples and RLPI-KFBY1, their average abundance was

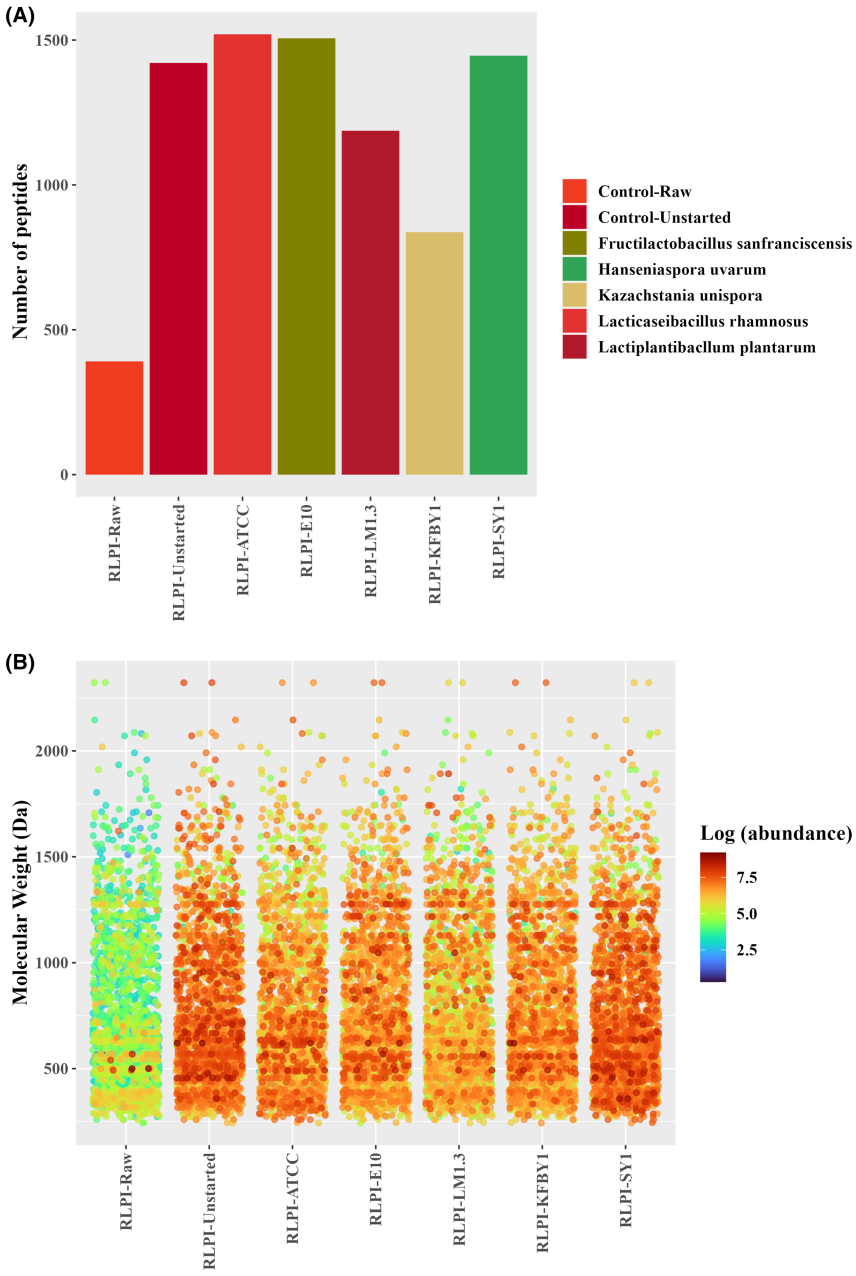

FIGURE 4 Peptidomic analyses of low molecular weight water soluble extracts (LMW-WSE) obtained from raw red lentils protein isolate (RLPI-Raw), RLPI without inoculum (RLPI-Unstarted) and Fermented-RLPI, which were incubated at 30°C for 8 days. Fermentation was done spontaneously (RLPI-Unstarted) or *Lactiplantibacillus plantarum* LM1.3 (RLPI-LM1.3), *Lacticaseibacillus rhamnosus* ATCC53103 (RLPI-ATCC), *Fructilactibacillus sanfranciscensis* E10 (RLPI-E10), *Kazachstania unispora* KFBY1 (RLPI-KFBY1) and *Hanseniaspora uvarum* SY1 (RLPI- SY1). Total number of different peptides found in each sample (A) and their distribution based on the molecular weight, employing a colour scale that transitions from blue to red to represent the Log abundance of each identified peptide within each sample (B).

substantially lower. RLPI-SY1 and RLPI-Unstarted were the richest sources of BPs compared with other treatments. The key BPs highly characterizing the RLPI-SY1 were ALEPDHR (Antioxidant), AVV (ACE-inhibitory), FFI (ACE-inhibitory), FGG (ACE-inhibitory), KVI (Antioxidant), LVL (ACE-inhibitory), LVR (ACE-inhibitory) and VVR (ACE-inhibitory). Among the three most abundant peptides in RLPI-SY1, the sequences LVR and LVL were never previously detected in substrates subjected to microbial fermentation (Hazato & Kase, 1986; Maruyama et al., 1989). KVI, also abundant in RLPI-SY1, was previously found only in dried bonito (Suetsuna Kunio, 1999). The high abundance of these BPs, together with other minor BPs, might account for the excellent functional properties observed in RLPI-SY1 during our screening.

While LAB, or in some cases moulds, have been extensively studied and documented in recent literature as biological tool to release BPs (Dhull et al., 2023; Garrido-Galand et al., 2021; Magro et al., 2019), the potential of *H. uvarum* SY1 emerged under the conditions of our study.

The main BPs highly present in RLPI-Unstarted were ALEPDHR, KAL (Antioxidant), LAE (ACE-inhibitory), and PLLR (Antioxidant). The other fermented samples showed different BPs values, consistently higher than the control and lower than the two previously mentioned samples (Table S3, Figure 5b,c). Correlation matrix based on spearman correlation coefficient were established to confirm the relationship between BPs and the screened bioactivities (Figure 6). The first correlation was with the

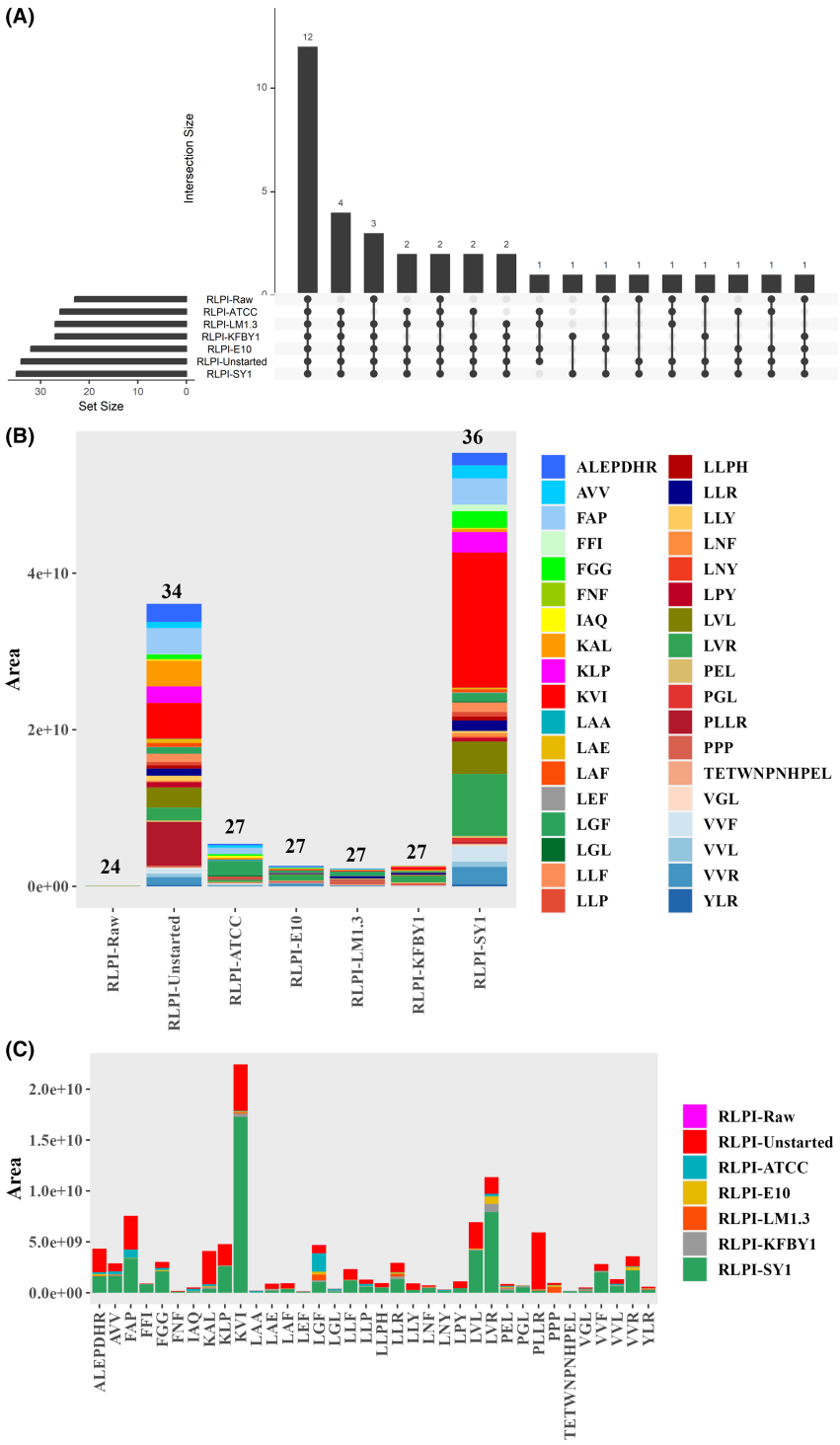

FIGURE 5 Peptidomic analyses of low molecular weight water soluble extracts (LMW-WSE) obtained from raw red lentils protein isolate (RLPI-Raw), RLPI without inoculum (RLPI-Unstarted) and Fermented-RLPI, which were incubated at 30°C for 8 days. Fermentation was done spontaneously (RLPI-Unstarted) or *Lactiplantibacillus plantarum* LM1.3 (RLPI-LM1.3), *Lacticaseibacillus rhamnosus* ATCC53103 (RLPI-ATCC), *Fructilactibacillus sanfranciscensis* E10 (RLPI-E10), *Kazachstania unispora* KFBY1 (RLPI-KFBY1) and *Hanseniaspora uvarum* SY1 (RLPI- SY1). Upset plot of the intersection of samples, sorted by identified BPs sharing 100% sequence homology with known bioactive peptides using BIOPEP UWM database, (dark circles in the matrix indicate sets that are part of the intersection) (A); relative quantification of BPs and distribution in the samples (B, C). The statistical analysis is shown in the Table S3.

36 BPs previously discovered for their ACE-inhibitory and antioxidant activities (Figure 6a). As expected, the majority of BPs demonstrated a negative strong correlation with the IC_{50} values (corresponding to ACE inhibition), with few exceptions such as IAQ, KAL, LAA, LGF and PLLR. Despite being classified for ACE inhibition in the literature, only PPP showed a substantially positive correlation with IC_{50} values. This finding prompts further investigation into the extent of biological activity by BPs already documented in the scientific literature. On the other hand, with the exception of IAQ, KAL, LAA, PLLP and PPP, all BPs were positively correlated with antioxidant activity. As previously reported, the LMW-WSE of *H. uvarum* SY1 exhibited potent in vitro antioxidant and antihypertensive activity. The peptides KVI, LVL and LVR were the most abundant peptides produced by RLPI-SY1 and resulted strongly associated with anti-ACE and antioxidant activity. Additionally, several peptides (e.g. ADELPDHR, FAP, FFI, KLP, LLP, LLPH, LNF and

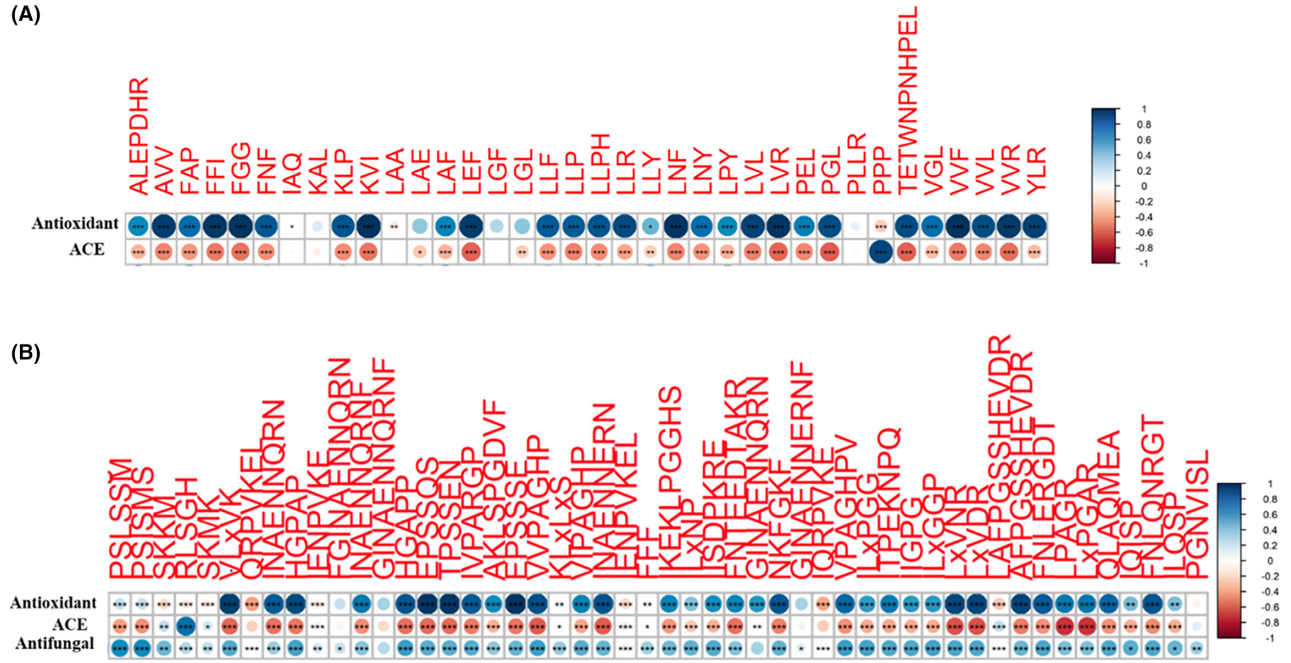

FIGURE 6 Spearman's rank correlation matrix between identified bioactive peptides (panel A) and top 50 un-identified peptides and functional bioactivities (panel B). Large and small circles indicate strong and weak correlations, respectively. Colours of the scale bar describe the type of correlation: 1 indicates a perfect positive correlation (dark blue) and −1 indicates a perfect negative correlation (dark red) between two microbial populations. The significance *p*-values were not corrected by FDR and were represented by (*) < 0.05, (**) < 0.01, (***) < 0.001.

LVR) were exclusively found in the fermented sample and not in the raw substrate. These peptides also exhibited a strong correlation ($p < 0.05$) with anti-ACE and antioxidant activity. To gain more insight, we further looked at the relationships between the antifungal assay results and the 50 most prevalent peptides (Figure 6b). 44 peptides were strongly correlated to radial growth rate inhibition against *P. roqueforti* P1. Within the fermented and unfermented samples, it was found that RLPI-SY1 exhibited the presence of all these peptides in varying quantities. Notably, PSSSE, EPSSQS, HGPAP, HGAPP, INAENNQRNF, PSLSSM, PSISMS, TPSSEN and VLVK were observed to have the highest abundance in RLPI-SY1 and were solely identified in the fermented RLPI. This opens the door to further investigation, such as the ex novo synthesis of these peptides followed by validation of the investigated bioactivity. BPs research is driven by the quest to uncover novel bioactive sequences that can expand the range of applications and benefits associated with BPs. Thus, the discovery of these novel potential antifungal sequences is critical for advancing BPs research.

CONCLUSION

Under the experimental conditions of our study, RLPI proved to be a suitable substrate for releasing BPs by assisted-starter fermentation. The effectiveness of the proteolytic system of the microorganisms investigated was species-dependent. In particular, the role of the yeast *H. uvarum* SY1, which had hitherto been little exploited for BPs release in food systems, emerged. Compared to RLPI-raw, fermentation with *H. uvarum* SY1 resulted in an increase in ABTS radical removal, ACE inhibition and antifungal activities, as detected in the low molecular weight water-soluble extracts. The increase was linked to the release of certain BPs, some of which were novel in raw RLPI or fermented matrices. Furthermore, 44 peptides were correlated to antifungal activity for the first time and will deserve further characterization.

AUTHOR CONTRIBUTIONS

Stefano Tonini: Conceptualization (equal); formal analysis (equal); investigation (equal); visualization (equal); writing – original draft (equal). **Ali Zein Alabiden Tlais:** Data curation (equal); formal analysis (equal); visualization (equal); writing – review and editing (equal). **Bruno Domingues Galli:** Investigation (equal). **Ahmed Helal:** Formal analysis (equal); methodology (equal). **Davide Tagliazucchi:** Formal analysis (equal); methodology (equal); writing – review and editing (equal). **Pasquale Filannino:** Writing – review and editing (equal). **Emanuele Zannini:** Writing – review and editing (equal). **Marco Gobbetti:** Funding acquisition (equal); supervision (equal). **Raffaella Di**

Cagno: Supervision (equal); writing – review and editing (equal).

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CONFLICT OF INTEREST STATEMENT

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.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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