**Advancement and prospects of study of bioactive peptides during food fermentation**

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In the current trend favoring plant-based foods over animal-based foods, pulses offer an alternative source of protein as well as bioactive peptides (BPs). We examined how proteins in a red lentils protein isolate (RLPI) break down during fermentation with different lactic acid bacteria and yeast strains*. Hanseniaspora uvarum* SY1 and *Fructilactobacillus sanfranciscensis* E10 were the most effective microorganisms in terms of protein hydrolysis*. H.uvarum* SY1 produced the highest levels of antiradical, ACE-inhibitory, and antifungal activities in the low molecular weight water soluble extracts (LMW-WSE). We analyzed the 2039 peptide sequences identified in the LMW-WSE using the BIOPEP UWM database, and 36 sequences matched with known BPs. Fermentation generated 12 peptides that were not present in raw RLPI. Furthermore, *H.uvarum* SY1 resulted in the highest quantities of BPs, particularly those with antioxidant and ACE-inhibitory properties. The peptides KVI, LVR, and LVL were identified for the first time in the fermented samples. Additionally, we found 44 new potential BPs that exhibited antifungal activity and are deserving of further investigation and characterization.

# **Avanzamento e prospettive di studio dei peptidi bioattivi durante la fermentazione di matrici alimentari**

Nella tendenza attuale che vede i cibi di origine vegetale preferiti rispetto a quelli di origine animale, i legumi offrono un'alternativa come fonte di proteine e di peptidi bioattivi (BPs). Abbiamo esaminato come le proteine dell'isolato proteico di lenticchie rosse (RLPI) possano essere idrolizzate durante la fermentazione condotta con diversi ceppi di batteri lattici e lieviti. *Hanseniaspora uvarum* SY1 e *Fructilactobacillus sanfranciscensis* E10 sono risultati i microrganismi più efficaci nell’idrolisi delle proteine. *H.uvarum* SY1 ha prodotto i livelli più elevati di attività anti-radicalica, ACE-inibitoria e antifungina negli estratti solubili in acqua a basso peso molecolare (LMW-WSE). Abbiamo analizzato le 2039 sequenze peptidiche identificate nei LMW-WSE utilizzando il database BIOPEP UWM, e 36 sequenze corrispondevano a BPs noti. La fermentazione ha generato 12 peptidi non presenti nella RLPI non fermentata. Inoltre, *H.uvarum* SY1 ha prodotto le quantità più elevate di BPs, in particolare quelli con proprietà antiossidanti e ACE-inibitorie. I peptidi KVI, LVR e LVL sono stati identificati per la prima volta nei campioni fermentati. Inoltre, abbiamo trovato 44 nuovi potenziali BPs che mostravano attività antifungina e meritano ulteriori indagini e caratterizzazioni.

**Key words**: Lactic acid bacteria; yeasts; antiradical; ACE-inhibitory; antifungal, bioactive peptides, high resolution tandem mass spectrometry.

**Introduction**

Bioactive peptides production through microbial fermentation is considered by several authors (Gobbetti et al.;2007 De Pasquale et al., 2020) to be one of the most effective non-thermal, green biotechnology to exploit the full biological potential of different protein sources. It is a process that meets the requirements of sustainability, innovation and functionality, but in order to be effective it must necessarily be monitored and designed to obtain the metabolic pathways of interest (Tlais et al., 2021). This report highlights the main results of the three years of research activities of my PhD in Food Engineering and Biotechnology.

In particular, we investigated the release of bioactive peptides from proteins during fermentation of red lentils protein isolate (RLPI) with different microbial strains: *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). The fermented LMW peptides extracts were tested for different bioactivities including antiradical, ACE-inhibitory, and antifungal activities and further identified via high resolution tandem mass spectrometry (UHPLC-HRMS2).

**Materials and methods**

Red lentils protein isolate (50 g) and water (100 g) were mixed to form a fermentable dough with a dough yield (DY) of 300. Glucose (1%, w/w) was added to the dough, and it was then singly fermented using pure cultures of LAB and yeast strains. After 24 hours, the cells were harvested by centrifugation at 10,000 rpm for 10 minutes at 4 °C, washed twice in 50 mM sterile potassium phosphate buffer (pH 7.0), and then inoculated into the RLPI dough. The final cell densities were approximately 7.0 Log CFU mL-1 for LAB and 5.0 Log CFU mL-1 for yeasts. The RLPI dough was fermented at 30 °C for 8 days. Two control samples were used: RLPI dough without bacterial inoculum and incubation (RLPI-Raw), and RLPI dough without inoculum but incubated at 30 °C (RLPI-Unstarted). To isolate the active peptide fraction, the water-soluble extract (WSE) was subjected to ultrafiltration with a molecular weight cut-off of less than 3 kDa, following the method described by Tagliazucchi et al. (2017) with some modifications. In this process, 15 mL of the sample was loaded into a Vivaspin®20 column with a 3000 MWCO-PES membrane (Sartorius, Italy) and centrifuged at 6000 rpm for 40 minutes. The resulting low molecular weight water-soluble extract (LMW-WSE) was used for further analysis. The identification of low molecular weight peptides in the LMW-WSE was performed using 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), following the method described by Martini et al. (2020). The MS data was first converted into a .mgf file and then processed using the MASCOT software (Matrix Science, Boston, MA, USA) for peptide sequencing and identification. The identification process employed the following parameters: no enzyme specified, peptide mass tolerance of ±5 ppm, fragment mass tolerance of ±0.1 Da, and variable modifications including Deamidation (NQ), oxidation (M), and phosphorylation (ST). A maximum of one post-translational modification was permitted in a single peptide. Only peptides identified with a significance threshold of P<0.05 were considered for further analysis.

**Results & Discussion**

The inhibitory effects of raw and fermented Red Lentils Protein Isolate (RLPI) on the mold *Penicillium roqueforti* P1, a common bread spoilage agent, were evaluated using peptide extracts (LMW-WSPE). All LMW-WSE derived from fermented samples significantly enhanced the inhibition of radial hyphal growth rate in *P. roqueforti* P1 compared to RLPI-Raw (12.5 ± 1.52%). Notably, RLPI-SY1 exhibited the highest inhibitory activity (approximately 60.4 ± 0.7%), followed by RLPI-Unstarted (60.1 ± 1.06%), RLPI-LM1.3 (56 ± 1.06%), and RLPI-KFBY1 (54.2 ± 0.78%). The lowest inhibition of radial growth, relative to the control, was observed in RLPI-ATCC (50 ± 0.85%) and RLPI-E10 (35.4 ± 1.13%) (Figure 1A). Previous studies have confirmed the involvement of lactic acid bacteria (LAB), particularly *Lp. plantarum* and *Lc. rhamnosus*, with proteolytic activities in generating antifungal bioactive peptides against various *Penicillium* spp. However, there is currently no available literature data on the potential of *H. uvarum*, which exhibited the strongest inhibitory effect on *P. roqueforti* P1, in releasing antifungal peptides.

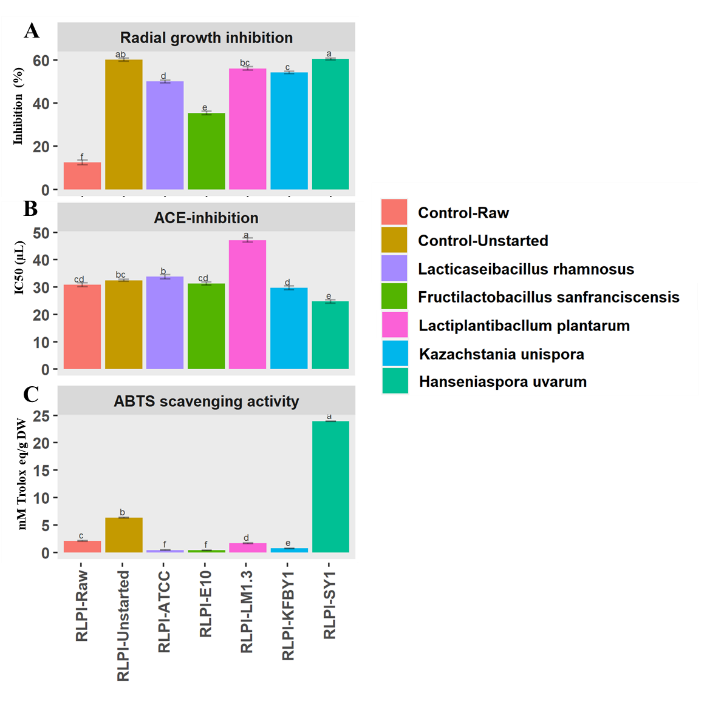
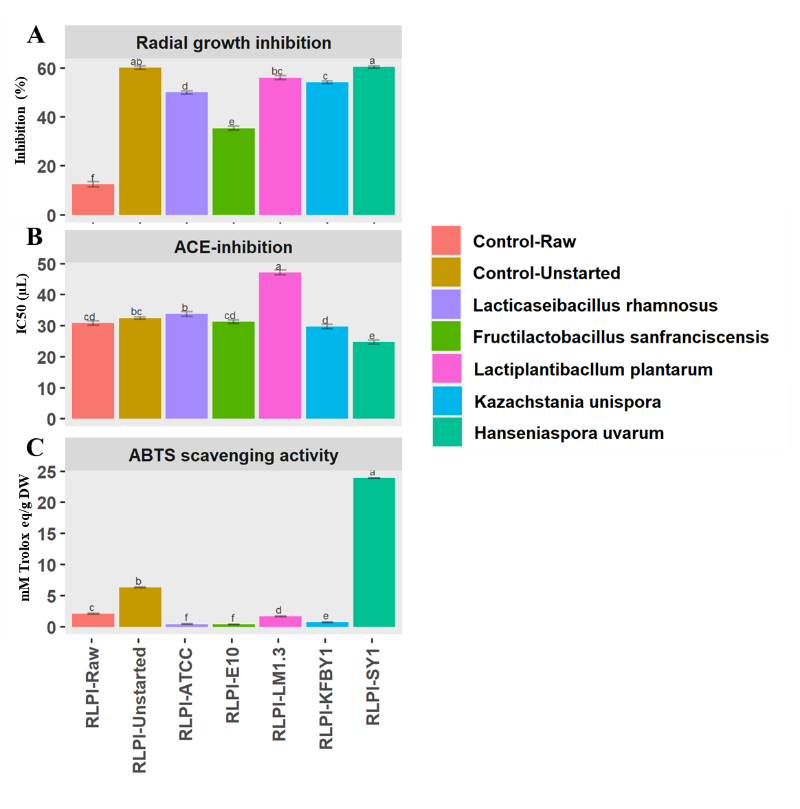
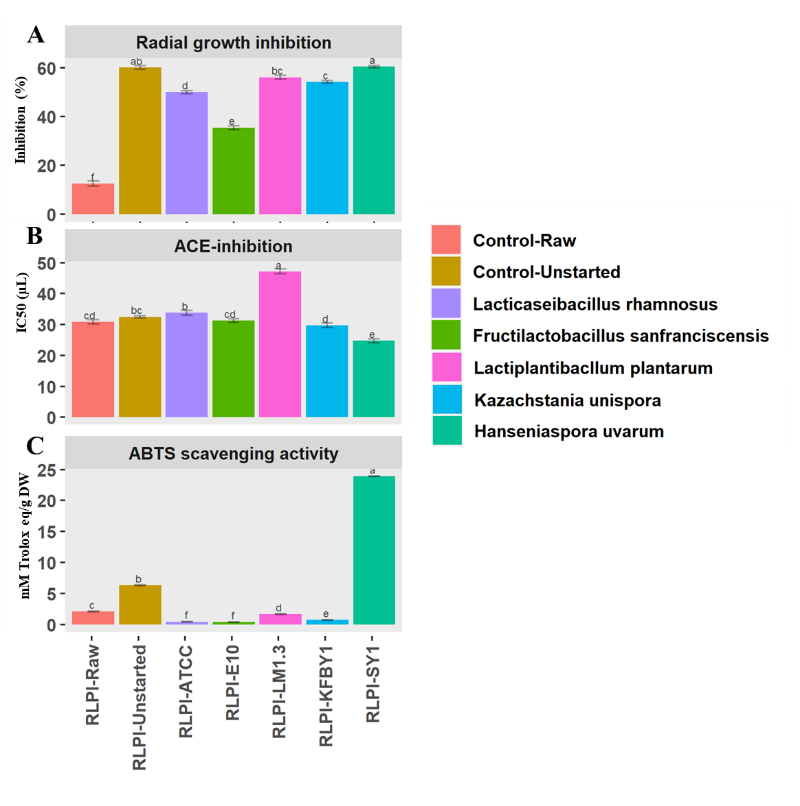
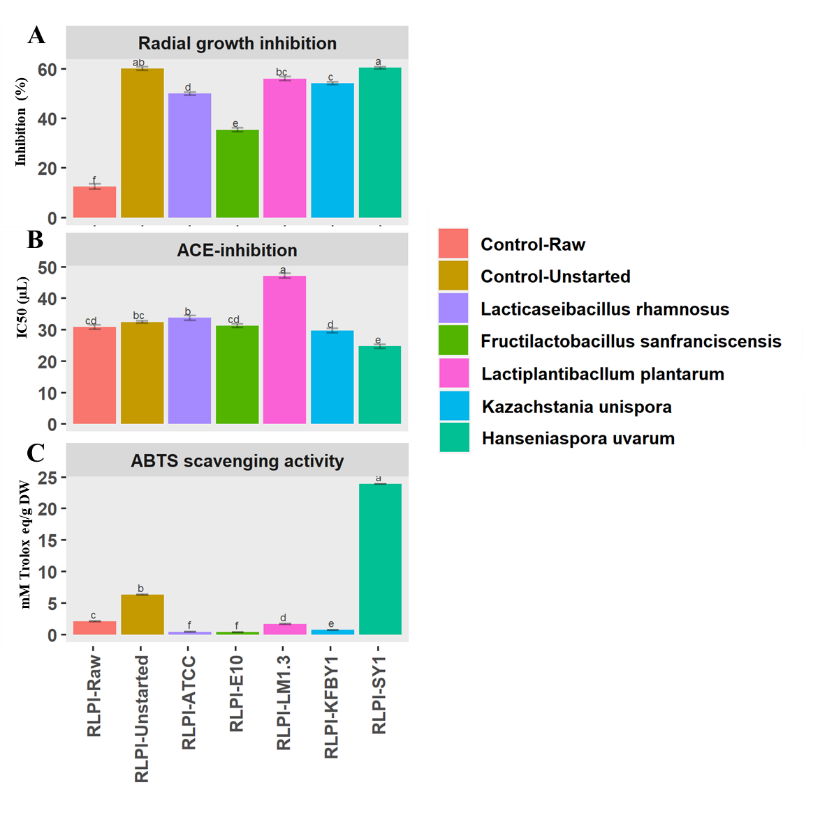
The renin-angiotensin system (RAS) plays a crucial role in regulating fluid balance and blood pressure, making it a significant metabolic process in cardiovascular homeostasis. Angiotensinogen, a protein primarily produced in the liver, is converted to angiotensin I (Ang I) by renin, an enzyme mainly secreted by the kidney. Angiotensin-converting enzyme (ACE), predominantly found in the lungs, converts Ang I to angiotensin II (Ang II), which has implications for human health (Marques et al., 2012). Evaluating the inhibitory effect on ACE activity is important for its potential impact on hypertension. In this study, an in vitro ACE-inhibitory activity assay was conducted to assess the antihypertensive potential of raw and fermented RLPI, and the results were reported as IC50, representing the amount of LMW-WSPE extract required to inhibit 50% of ACE activity. Among the samples, only RLPI fermented with *H. uvarum* SY1 (RLPI-SY1) exhibited significant (P < 0.05) ACE inhibition, with the lowest IC50 value (24.75 ± 0.77 µL) compared to RLPI-Raw (30.79 ± 0.87 µL) (Figure 1B). RLPI-Unstarted, RLPI-E10, and RLPI-KFBY1 showed similar IC50 values without statistical significance (P > 0.05) compared to RLPI-Raw. Notably, started fermentation had a significant negative effect (P < 0.05) on ACE inhibition in RLPI-LM1.3 (47.13 ± 0.97 µL) and RLPI-ATCC (33.76 ± 0.92 µL) (Figure 1B). Previous research has explored the potential inhibitory effect of red lentil protein hydrolysates, revealing the generation of bioactive peptides in RLPI hydrolyzed with commercial trypsin (Boye et al., 2010). Although the proteolytic systems of various lactic acid bacteria have been utilized to produce bioactive peptides with ACE-inhibitory activity through fermentation, none of the LAB starters used in our study were able to release anti-ACE peptides. In contrast, our findings demonstrated the effectiveness of *H. uvarum* SY1 in suppressing ACE activity. While yeast cells have been investigated as a source of bioactive peptides with ACE-inhibitory properties (Mirzaei et al., 2021), their use as starters to induce protein hydrolysis and release anti-ACE peptides has not been previously reported.

Antioxidant peptides have gained attention as a promising strategy for preventing oxidative stress and preserving food quality, thereby reducing economic losses in the food industry and improving public health (Chakrabarti et al., 2014). In this study, the ABTS radical scavenging capacity of LMW-WSE from raw and fermented RLPI was evaluated. Compared to RLPI-Raw (2.12 ± 0.06 mM Trolox eq. g-1 DW), a significant increase (P < 0.05) in the ABTS radical scavenging capacity was observed in LMW-WSE 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 even after heat treatment at 100 °C for 5 minutes. Interestingly, the lactic acid fermentation had an unexpected negative effect on the antioxidant activity of RLPI (Figure 1C). This finding contradicted previous studies that highlighted the potential role of *Lp.* *plantarum* strains in cow's milk and *Lc. rhamnosus* in releasing antioxidant-rich hydrolysates and peptides (Aguilar-Toalá et al., 2017; Solieri et al., 2015).

In order to assess the impact of fermentations on peptide release, the peptide profile was examined using HPLC-HRMS analysis. Over the past few years, HRMS-based peptidomics analysis has emerged as a reliable and sensitive method for comprehensive mapping of the peptidome present in various samples. MS-based peptidomics analysis is particularly suitable for monitoring the abundance of known peptides in samples; however, its application in discovering novel bioactive peptides presents challenges due to the presence of numerous degradation products and inactive precursors (Aydoğan, 2020). The analysis revealed a total of 2039 distinct peptides across all samples, with only 391 peptides detected in RLPI-Raw. The variation in proteolytic activity associated with different starters had a significant impact on the quantity and diversity of peptides identified (Figure 2A). The use of lactic acid bacteria (LAB) led to the highest increase in the number of peptides, with RLPI-ATCC (1520 peptides) and RLPI-E10 (1506 peptides) exhibiting the greatest peptide diversity among all samples. Among yeast fermentations, RLPI-SY1 showed the highest diversity of peptide substrates, following RLPI-ATCC and RLPI-E10, while RLPI-KFBY1 displayed the lowest diversity. The influence of spontaneous fermentation on proteolytic activity was evident in RLPI-Unstarted, which showed a high number of distinct peptides (1421 peptides). The identified peptides in the analyzed samples were compared to known bioactive peptides (BPs) sequences using the BIOPEP UWM database. This database, which contains over 4600 BP sequences, has become popular in the field of food and nutrition science as a valuable source of data on these molecules, which are of great interest for potential use as functional food ingredients and nutraceutical applications (Minkiewicz et al., 2019). Most of the peptides found in both raw and fermented RLPI were related to parental proteins from chickpeas and a few other legumes. Out of the 2039 peptide sequences identified, a total of 36 peptides showed 100% identity with previously identified and validated BP sequences (Figure 2). The majority of these discovered BPs were associated with plant proteins. It is worth noting that most research on BPs has primarily focused on precursor proteins from dairy, meat, and fish, with a smaller portion dedicated to plant-based proteins (Bhat et al., 2015). Among these 36 BPs, 73% exhibited ACE-inhibitory activity, 22% had antioxidant activity, and 5% were associated with other bioactivities such as DPPIV-inhibition and antidiabetic properties. Thirteen of these BPs (ALEPDHR, FAP, FFI, KLP, LLP, LLPH, LNF, LVR, PLLR, PPP, TETWNPNHPEL, VVR, YLR), which were mainly ACE-inhibitory and antioxidant, were exclusively found in the fermented samples in varying amounts. Although the intensity of peaks varied, RLPI-SY1 had the highest number of BPs (36), followed by RLPI-Unstarted (35), while the remaining samples exhibited lower numbers ranging from 27 to 32. Furthermore, only 12 bioactive peptides were common among all the samples, with RLPI-Raw having the lowest diversity (Figure 2B). The impact of fermentation on the abundance of BPs was evident from the relative quantification analysis (Figure 2B and C). With few exceptions, each BP showed a considerable increase in peptide abundance compared to RLPI-Raw. RLPI-SY1 resulted in the highest cumulative intensity of BPs, followed by RLPI-Unstarted. Although LAB-fermented samples and RLPI-KFBY1 contained a higher number of BPs, their average abundance was relatively lower. RLPI-SY1 and RLPI-Unstarted were the richest sources of BPs compared to other treatments. The main bioactive peptides (BPs) that highly characterized 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). It is noteworthy that KVI, LVR, and LVL, which were the three most abundant peptides, were not previously obtained through fermentation. The presence of these BPs, along with other minor BPs, may contribute to the excellent functional properties observed in RLPI-SY1 during our screening. The main BPs highly present in RLPI-Unstarted were ALEPDHR, KAL (antioxidant), LAE (ACE-inhibitory), and PLLR (antioxidant). The other fermented samples exhibited different values of BPs, consistently higher than the control and lower than the two previously mentioned samples (Figure 2B and C). A correlation matrix based on the Spearman correlation coefficient was established to confirm the relationship between BPs and the screened bioactivities. The first correlation was made with the 36 BPs previously identified for their ACE-inhibitory and antioxidant activities. As expected, the majority of BPs showed a strong negative correlation with the IC50 values (indicative of ACE inhibition), with a 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 IC50 values. On the other hand, except for 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, which were the most abundant peptides produced by RLPI-SY1, were strongly associated with anti-ACE and antioxidant activity. Additionally, several peptides (e.g., ADELPDHR, FAP, FFI, KLP, LLP, LLPH, LNF, and LVR) were exclusively found in the fermented sample and not in the raw substrate. These peptides also showed a strong correlation (P < 0.05) with anti-ACE and antioxidant activity. To gain more insight, we further investigated the relationships between the antifungal assay results and the 50 most prevalent peptides. Forty-four peptides showed a strong correlation with the inhibition of radial growth rate against P. roqueforti P1. Among the fermented and unfermented samples, it was found that RLPI-SY1 contained all these peptides in varying quantities. Notably, PSSSE, EPSSQS, HGPAP, HGAPP, INAENNQRNF, PSLSSM, PSISMS, TPSSEN, and VLVK were the most abundant peptides in RLPI-SY1 and were solely identified in the fermented RLPI. This discovery opens the door to further investigation, such as the ex-novo synthesis of these peptides, followed by validation of their bioactivity.

**Conclusions**

Under the experimental conditions of our study, we found that RLPI (rice protein isolate) was a suitable substrate for the release of bioactive peptides (BPs) through assisted-starter fermentation. The effectiveness of the proteolytic system of the microorganisms we investigated varied depending on the species. In particular, the yeast *H. uvarum* SY1, which has been underutilized in the release of BPs in food systems, showed promising results. Fermentation with H. uvarum SY1 led to an increase in ABTS-radical removal, ACE inhibition, and antifungal activities compared to raw RLPI. These activities were detected in the low molecular weight water-soluble extracts. The increase in activities was attributed to the release of specific BPs, some of which were previously unidentified in raw RLPI or fermented matrices. Additionally, we discovered 44 peptides that were correlated with antifungal activity for the first time, highlighting the need for further characterization of these peptides.



A picture containing text, screenshot, diagram, plot

Description automatically generated**Figure 1.** In vitro determination of radial growth inhibition against *Penicillium roqueforti* P1 (panel A), ACE-inhibitory (B), and ABTS scavenging activity of low molecular weight water soluble extracts (LMW-WSE) obtained from fermented and unfermented red lentils protein isolate.

**Figure 2**. Peptidomic analyses of low molecular weight water soluble extracts (LMW-WSE) obtained from raw and fermented red lentils protein isolate. Total number of different peptides found in each sample (A); 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) (B); relative quantification of BPs and distribution in the samples (C and D).

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