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# Peptidomics and biological activity analysis of *in vitro* digested Parmigiano Reggiano cheese at different ripening stages

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ABSTRACT

The relationships among Parmigiano Reggiano (PR) cheese ripening, *in vitro* digestion, and biological activity have been investigated. PR cheese samples at different ripening time (from 12 to 30 months) were collected from two dairies and *in vitro* digested. No effect of ripening was found when the total peptide profiles were considered. Nevertheless, ripening affected the biological activities and the bioactive peptide profiles of *in vitro* digested PR. The long-ripened PR samples showed higher ACE-inhibitory and DPP-IV-inhibitory activities after *in vitro* digestion. The ACE-inhibitory activity of *in vitro* digested PR samples was positively correlated with the relative amount of potent ACE-inhibitory peptides whereas DPP-IV-inhibitory activity with protein hydrolysis. Some bioactive peptides that, according to the literature, exhibit proven *in vivo* bioactivity and bioavailable in humans were detected, suggesting their potential role in the protective effects on human health resulting from cheese consumption. PR samples at different ripening time may have potentially different effects on human health.


## 1. Introduction

Fermentation is one of the most ancient preservation techniques used in the human history and fermented dairy food consumption by humans date back between 7000 and 10,000 years ago (Bintsis & Papademas, 2022; Marco et al., 2021). Dairy products account for the 10.6% of dietary energy intake in Western diet with fermented dairy products providing the 5.8% of total dietary energy (Cordain et al., 2005). In addition to their high nutritional value, fermented dairy products have recently gained considerable interest for their presumed health-promoting properties (González et al., 2019; Rul et al., 2022; Tagliazucchi, Martini, & Solieri, 2019). Beneficial compounds found in fermented dairy products include vitamins (mainly vitamins D, A, K, and B12), minerals (such as calcium and magnesium), bioactive lipids and bioactive peptides (Tagliazucchi et al., 2019; Thorning et al., 2017). Furthermore, fermented dairy products may enclose specific probiotic lactic acid bacteria that may have a positive impact on intestinal health and function (Gou, Zhang, Ren, Li, & Zhang, 2022).

Among fermented dairy products, cheese is one of the most popular and the global consumer demand for cheese is continuously increasing with an expected rise in the cheese market of about 3–7% annually. Despite the high salt and saturated fats content, the consumption of cheese and, in particular, of long-ripened cheeses is claimed by many nutritional guidelines to sustain a healthy diet.

Actually, several recent studies suggested that cheese consumption may be associated with a reduced risk of onset of cardiovascular diseases (including coronary heart disease, hearth failure and ischemic stroke) as well as of type-2 diabetes (Alexander et al., 2016; Gao et al., 2013; Zhang et al., 2023).

Parmigiano Reggiano (PR) is a worldwide known hard and long-ripened Italian cheese made from raw milk (a blend of whole and partially skimmed raw milk) supplemented with natural whey starter (composed by homofermentative and thermophilic starter lactic acid bacteria) (Martini et al., 2024; Tagliazucchi et al., 2020). PR is a Protected Designation of Origin (PDO) cheese, exclusively produced in a confined geographic area in Northern Italy and ripened for at least 12 months (Tagliazucchi et al., 2020). During PR production and, especially, ripening different biochemical events occur, the most important of which is proteolysis (McSweeney, 2004). The first proteolytic events take place during cheese manufacturing where chymosin, endogenous milk proteolytic enzymes, and starter lactic acid bacteria begin to break down milk caseins releasing large and intermediate-size peptides (Sforza et al., 2012). After curd brining, the starter lactic acid bacteria population

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is quickly replaced by non-starter lactic acid bacteria mainly belonging to the Lacticaseibacillus group, including *Lacticaseibacillus rhamnosus*, *Lacticaseibacillus paracasei*, and *Lacticaseibacillus zeae* (Bottari et al., 2020; Martini et al., 2024).

Many of these non-starter lactic acid bacteria have a complex proteolytic system which includes cell-envelope proteinases and cytoplasmic peptidases able to further cleave casein-derived oligopeptides into low molecular weight peptides and amino acids (Tagliazucchi et al., 2019). Live cells and their free enzymes released after cellular lysis are responsible for continuous proteolysis occurring during ripening. Therefore, the peptide profile of PR continuously evolves and changes during ripening and the presence of peculiar lactic acid bacteria population may affect the peptide profile of PR during ripening (Bottari et al., 2020; Martini et al., 2024).

Among the pool of peptides found in PR cheeses at different ripening time, some of them presented specific bioactivities which encompass antioxidant activity, angiotensin-converting enzyme (ACE) inhibitory activity, dipeptidyl-peptidase IV (DPP-IV) inhibitory activity and anti-microbial activity (Bottari et al., 2020; Martini, Conte, & Tagliazucchi, 2020; Martini, Solieri, Cattivelli, Pizzamiglio, & Tagliazucchi, 2021; Martini et al., 2024). Most of the identified bioactive peptides in PR cheese were ACE-inhibitory peptides and some of them, such as the peptide VPP, IPP, LHLPLP, HLPLP and AYFYPEL, have proven anti-hypertensive activity *in vivo* in human or animal models (Bernabucci, Catalani, Basiricò, Morera, & Nardone, 2014; Martini et al., 2020). Some bioactive peptides identified in PR cheese have been found bioavailable in human volunteers after the consumption of dairy products (Caira et al., 2022). Moreover, it has been recently demonstrated that the consumption of PR cheese led to a transitory and dose-dependent reduction in systolic blood pressure in spontaneously hypertensive rats (Basiricò et al., 2022).

Besides the presence of bioactive peptides in PR cheese, it is known that gastro-intestinal digestion may modulate the bioactive peptide profile of cheeses (Castellone et al., 2022; Helal, Cattivelli, Conte, & Tagliazucchi, 2023a; Martini et al., 2020; Stuknyte, Cattaneo, Masotti, & De Noni, 2015). Bioactive peptides can be degraded during gastro-intestinal digestion releasing inactive or still active sequences or new bioactive peptides encrypted in proteins or oligopeptides can be released following gastro-intestinal digestion (Castellone et al., 2022; Helal et al., 2023a; Martini et al., 2020; Stuknyte et al., 2015). In two previous studies, 21 and 52 bioactive peptides were identified in digested PR cheeses at different ripening times (Castellone et al., 2022; Martini et al., 2020). However, the effect of *in vitro* gastro-intestinal digestion on the biological activities of PR cheese and the correlation between the specific bioactive peptides and the related biological activities have only been poorly investigated. Therefore, the present study was designed to unravel the effect of *in vitro* gastro-intestinal digestion on the bioactive peptide profiles and biological activities of PR cheeses at different ripening time. Possible relationships between the bioactive peptide profiles and the associated biological activities have been also investigated.

## 2. Materials and methods

### 2.1. Materials and cheese sampling

Enzymes for *in vitro* gastro-intestinal digestion as well as reagents for the biological activity assays were supplied by Sigma–Aldrich (Milan, Italy). Solvents for high-resolution mass spectrometry experiments were purchased from Biorad (Hercules, CA, USA). Ultrafiltration filters with a molecular weight cut-off of 3 kDa were sourced from Millipore (Milan, Italy).

Cheese samples were collected from two different dairies located in the province of Reggio Emilia (Italy), referred to as C and L, respectively. From each dairy, PR samples at 12 and 24 months of ripening were collected in the first sampling. After six months, PR samples at 18 and 30 months of ripening were collected from the same wheels. A detailed description of sampling was reported in Martini et al. (2024). The alphanumeric sample code consisted of a letter that identified the dairy followed by a number that identified the ripening time.

### 2.2. In vitro *gastro-intestinal digestion of PR cheese samples*

*In vitro* gastro-intestinal digestion of PR cheese samples was performed following the INFOGEST 2.0 protocol (Brodkorb et al., 2019). Simulated salivary (SSF), gastric (SGF) and intestinal (SIF) fluids were prepared according to the INFOGEST 2.0 protocol. Salivary phase was carried out by mixing 1 g of grated PR with 1 mL of SSF containing 150 U $mL^{-1}$ of human salivary α-amylase and incubating the samples at 37 °C for 2 min in a rotating wheel (10 rpm). Next, for the gastric step, 2 mL of SGF (containing 4000 U $mL^{-1}$ of porcine pepsin and 120 U $mL^{-1}$ of porcine gastric lipase) were added, the pH corrected at 3 and the samples further incubated at 37 °C for 120 min in a rotating wheel (10 rpm). Finally, the intestinal step consisted in the addition of 4 mL of SIF (containing 20 mmol $L^{-1}$ of bile salts), correcting the pH at 7.5 and incubating the samples at 37 °C for 30 min in a rotating wheel (10 rpm). Finally, the digestion was completed by adding porcine pancreatin (final concentration in the digestive system based on trypsin activity of 100 U $mL^{-1}$) and incubating the samples at 37 °C for 120 min in a rotating wheel (10 rpm). At the end of the digestion, the samples were boiled for 5 min to inactivate the enzymes, centrifuged (20 min, 10,000 × *g*, 4 °C), and the supernatants stored at −20 °C before analysis.

A control digestion with water instead cheese was carried out to consider the possible interferences of the digestive system in the assays.

Digestions were performed in triplicate for each PR sample.

### 2.3. *Evaluation of protein hydrolysis and biological activities in* in vitro *gastro-intestinal digested PR cheese samples*

Protein hydrolysis in *in vitro* digested PR samples was assessed by measuring the amount of free amino groups by using the TNBS assay (Adler-Nissen, 1979). The data are reported as mmol of leucine equivalents in 100 g of PR cheese. The values were corrected for the contribution of the control digestion. Protein hydrolysis was also determined in water-soluble peptide fractions of PR cheese samples before digestion as previously described (Helal et al., 2023a).

Biological activity analysis were carried out on the supernatants of the *in vitro* digested samples after extraction of peptide fractions by ultrafiltration at 3 kDa cut-off.

The antioxidant activity of peptide fractions from *in vitro* digested PR cheeses was determined through the ABTS (2,2-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)) assay as previously described (Re et al., 1999). Results were reported as mg of ascorbic acid 100 $g^{-1}$ of cheese.

The ability of *in vitro* digested PR cheese peptide fractions to inhibit the angiotensin-converting enzyme (ACE) was evaluated by a spectrophotometer method using the tripeptide N-[3-(2-furyl) acryloyl]-L-phenylalanylglycyl-glycine (FAPGG) as a substrate and following the method described in Solieri et al. (2022).

The inhibitory activity of *in vitro* digested PR cheese peptide fractions towards the enzyme dipeptidyl-peptidase-IV (DPP-IV) was determined by applying the procedure reported in

Tagliazucchi, Martini, Shamsia, Helal, and Conte (2018) and using the peptide glycine-proline-*p*-nitroanilide as substrate.

For both the assays, the results were reported as $IC_{50}$ values, defined as the amount of cheese (mg of cheese $mL^{-1}$) needed to inhibit the enzymatic activity by 50%. The calculation of $IC_{50}$ was carried out as previously described (Helal & Tagliazucchi, 2023b).

### 2.4. Peptidomics analysis of in vitro digested PR cheese peptide fractions and label-free MS peak quantification

Peptidomics analysis of *in vitro* digested PR cheese samples peptide fractions were carried out by high-resolution mass spectrometry as previously described (Martini et al., 2021). Briefly, peptides were firstly separated with a UHPLC system (UHPLC Ultimate 3000 separation module, Thermo Scientific, San Jose, CA, USA) equipped with a C18 column (Acquity UPLC HSS C18 reversed phase, 2.1 × 100 mm, 1.8 μm particle size, Waters, Milan, Italy). Then separated peptides were sent to the high-resolution mass spectrometer (Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer; Thermo Scientific, San Jose, CA, USA) for their identification.

The two solvents used as mobile phase were water with 0.1% of formic acid (solvent A) and acetonitrile with 0.1% of formic acid (solvent B).

The gradient started at 2% of solvent B, and increased in 20 min—27% of solvent B. The solvent B concentration then reached the 90% in 5 min and this condition was then kept for further 3 min before to return to the initial conditions. The elution was carried out with flow rate of 0.3 mL $min^{-1}$.

The full mass spectrometry and tandem mass spectrometry parameters are reported in Martini et al. (2021).

Peptide sequencing was performed by using the Mascot software as previously reported (Martini et al., 2021). Firstly, the raw files from the mass spectrometer were converted in the format Mascot generic files (.mgf). Next, for peptide identification the following parameters were used: enzyme, none; peptide mass tolerance, ±5 ppm; fragment mass tolerance, ±0.12 Da; variable modification, oxidation (M), deamidation (NQ) and phosphorylation (ST); maximal number of post-translational modifications permitted in a single peptide, 4. Peptide identification was deemed correct with a significance threshold of $P < 0.05$.

For semi-quantitative analysis, the lists of peptides identified by Mascot were analyzed by Skyline (v23.1) using the protocol for label-free MS peak quantification previously described (Dallas & Nielsen, 2018; MacLean et al., 2010). A complete description of Skyline data analysis is reported in Helal et al. (2023b).

### 2.5. Bioactive peptide identification and semi-quantitative analysis

For the identification of bioactive peptides, the lists of identified peptides were analyzed with the Milk Bioactive Peptide Database (MBPDB, http://mbpdb.nws.oregonstate.edu/) (Nielsen, Beverly, Qu, & Dallas, 2017). Only peptides with 100% homology with previously reported biological activities were included in the bioactive peptide list. Skyline data were next used for semi-quantitative analysis of identified bioactive peptides.

### 2.6. Statistical analysis

Significant differences ($P < 0.05$) among *in vitro* digested cheeses were assessed by one-way ANOVA with Tukey post-test. Chemometrics analysis was carried out by using the online software MetaboAnalyst 5.0 (Xia, Sinelnikov, Han, & Wishart, 2015) to correlate peptide, bioactive peptides and biological activity data with cheese ripening. Data were normalized by median and mean centering before principal component analysis (PCA).

## 3. Results and discussion

### 3.1. Evaluation of protein hydrolysis in cheese and in vitro digested samples

Before digestion, the quantity of free amino groups varied depending on the ripening time (Fig. 1A). In the samples from both L and C dairies, the quantity of free amino groups remained constant up to 18 months of ripening and then increased significantly at 24 months ($P < 0.05$) and remained stable ($P > 0.05$) at 30 months. No significant differences were found between dairies at any ripening time except for PR samples at 24 months of ripening. Previous studies confirmed that proteolysis degree increases as a function of cheese ripening (Bütikofer, Meyer, Sieber, Walther, & Wechsler, 2008; Martini et al., 2020; Öztürk & Akın, 2021).

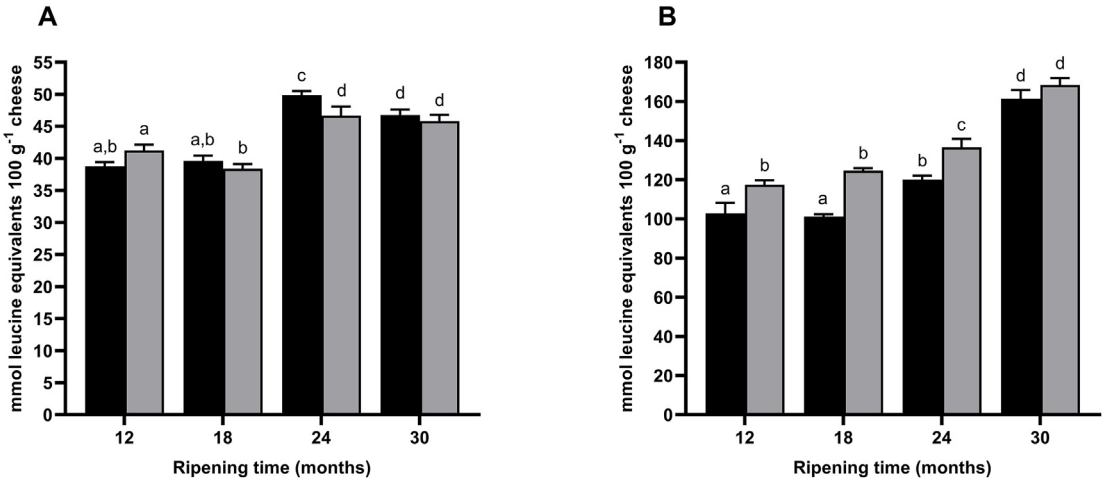


**Fig. 1.** Effect of ripening (A) and *in vitro* digestion (B) on protein hydrolysis of Parmigiano Reggiano cheeses at different ripening times. The numbers on the *x*-axis indicated the months of ripening. Black bars identified samples from dairy C whereas dark-gray bars from dairy L. Proteolysis was quantified by using the TNBS assay. The data are expressed as mmol of leucine equivalent 100 $g^{-1}$ of cheese. The *in vitro* digestion data were corrected for the contribution of the control digestion. Values are means of three assay replications ± standard deviation (SD). Different letters among samples denote significant differences ($P < 0.05$).

During cheese ripening, caseins are hydrolyzed by the activity of proteases and peptidases released from starter lactic acid bacteria after cell lysis and from non-starter lactic acid bacteria still alive in the ripened cheese (Tagliazucchi et al., 2019).

As expected, *in vitro* gastro-intestinal digestion increased the extent of protein hydrolysis, measured as amount of free amino groups, in all the tested PR samples at different ripening times (Fig. 1B). The amount of free amino groups released from PR samples after *in vitro* digestion was strongly influenced by ripening. It remained constant ($P > 0.05$) for both the dairies in *in vitro* digested PR samples at 12 and 18 months of ripening, then a significant increase was detected in *in vitro* digested PR samples at 24 months of ripening ($P < 0.05$). As depicted in Fig. 1B, in both the dairies the concentration of free amino groups in PR samples at 30 months of ripening was significantly higher than that released from the samples at 12, 18 and 24 months of ripening ($P < 0.05$). These results agree with those obtained by Martini et al. (2020) in PR samples at different ripening stages.

### 3.2. Biological activity analysis

The samples obtained at the end of the *in vitro* gastro-intestinal digestion were subjected to ultrafiltration to isolate peptides with a molecular weight lower than 3 kDa. The different peptide extracts were then characterized for their biological activity profile. The variation in antioxidant activity during the digestion of the different samples was followed through the ABTS assay, the results of which are reported in Fig. 2A. All the *in vitro* digested PR samples showed ABTS radical scavenging activity, but with some differences depending on dairy and ripening time. Digested PR samples obtained from dairy L exhibited similar antioxidant activities, regardless of ripening time ($P > 0.05$). *In vitro* digested PR samples from dairy C did not show any significant differences from each other in antioxidant activity after 12, 18 and 24 months ($P > 0.05$), whereas the *in vitro* digested PR sample at 30 months of ripening displayed significantly higher values compared to the 12, 18, and 24 month-ripened samples. When dairies C and L were compared to each other, *in vitro* digested PR samples from dairy L exhibited significantly higher antioxidant activity values after 12, 18 and 24 months of ripening in comparison with dairy C samples at the same ripening times. However, no significant differences were found between the two dairies after 30 months of ripening. A similar ripening time-dependence was already found for *in vitro* digested Ras cheese at 1, 3 and 6 months of ripening, although the antioxidant activity of *in vitro* digested PR samples was lower than that of *in vitro* digested Ras cheese (Helal et al., 2023a).

Dose-dependent ACE inhibitory activity was observed in all the peptide fractions of the digested samples (Fig. 2B). The hydrolyzates produced by the action of digestive enzymes in samples C at 24 and 30 months of ripening showed the highest ACE-inhibitory activity with $IC_{50}$ values of 0.49 ± 0.04 and 0.88 ± 0.02 mg of cheese $mL^{-1}$, respectively. In general, the $IC_{50}$ of *in vitro* digested PR samples from the two different dairies followed the same trend as a function of ripening. In this sense, ripening positively affected the ACE-inhibitory activity after *in vitro* digestion with the sample at 30 months of ripening showing the highest inhibitory potency. Similarly, in Ras and Cheddar cheeses the ACE-inhibitory activities of *in vitro* digested samples have been found to increase as a function of ripening (Helal et al., 2023a; Shaukat et al., 2022). Moreover, the ACE-inhibitory activity of digested PR samples from dairy C at 24 and 30 months of ripening was significantly higher ($P < 0.05$) than those measured in digested Ras and Cheddar cheeses (Helal et al., 2023a; Shaukat et al., 2022).

Similarly to what was previously observed for Ras cheese (Helal et al., 2023a), ripening time positively affected the DPP-IV-inhibitory activity of *in vitro* digested PR samples as highlighted by the decrease in $IC_{50}$ values against DPP-IV enzyme (Fig. 2C). In dairies C and L, the DPP-IV-inhibitory activities of samples after *in vitro* digestion increased up to 24 months of ripening and then remained constant in the samples after 30 months of ripening. The greatest inhibitory activity was detected in dairy L sample at 30 months of ripening and in dairy C sample at 24 months ($IC_{50}$ of 1.52 ± 0.08 and 1.91 ± 0.16 mg of cheese $mL^{-1}$, respectively). Very few studies have investigated the DPP-IV inhibitory activity of cheese after *in vitro* gastro-intestinal digestion. In this regard, PR samples had higher DPP-IV-inhibitory activity than Ras cheese after *in vitro* digestion (Helal et al., 2023a).

### 3.3. Peptidomics analysis

The peptidomics analysis enabled the identification of 415 unique peptides considering all the *in vitro* digested PR samples (see Supplementary Table S1 for the complete list of identified peptides). Castellone et al. (2022) analyzed the peptide profiles of 72 PR samples at different ripening times subjected to *in vitro* gastro-intestinal digestion and identified 105 different peptides. In another study the peptidomics profile of *in vitro* digested PR cheeses at 12, 18 and 24 months of ripening allowed the identification of 469 individual peptides (Martini et al., 2020).

By count, there were no substantial differences in the peptide numbers among the digested PR samples with a range of identified peptides between 373 and 385 (Fig. 3A). Most of the identified

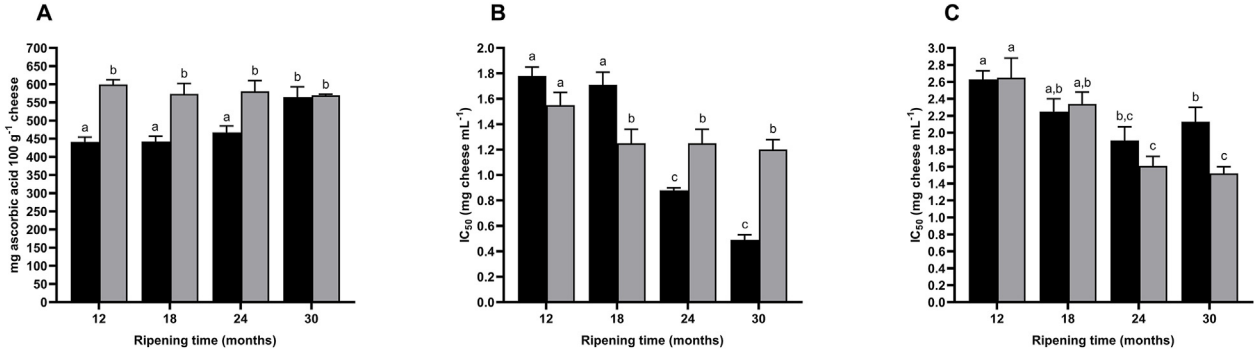


**Fig. 2.** Biological activity analysis of Parmigiano Reggiano cheeses at different ripening times after *in vitro* gastro-intestinal digestion. Biological activity analysis was performed on the low molecular weight peptide fractions (<3 kDa) obtained from the *in vitro* digested Parmigiano Reggiano cheese samples. The numbers in the *x*-axis indicated the months of ripening. Black bars identified samples from dairy C whereas dark-gray bars from dairy L. (A) Antioxidant activity determined with the ABTS assay. (B) ACE-inhibitory activity. (C) and DPP-IV-inhibitory activity. The *in vitro* digestion data were corrected for the contribution of the control digestion. Values are means of three assay replications ± standard deviation (SD). Different letters among samples in the same assay denote significant differences ($P < 0.05$).

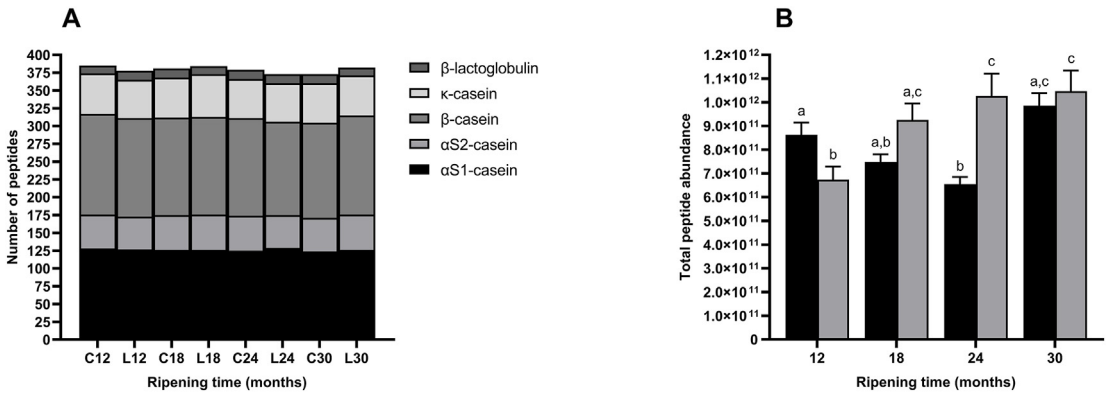


**Fig. 3.** Number of peptides *per* protein (A) and total peptide abundance (B) in *in vitro* digested Parmigiano Reggiano cheese at different ripening times. Analysis was performed on the low molecular weight peptide fractions (<3 kDa) obtained from the *in vitro* digested Parmigiano Reggiano cheese samples. (A) Number of peptides identified in *in vitro* digested Parmigiano Reggiano samples. (B) Total peptides abundance in *in vitro* digested Parmigiano Reggiano cheese samples. Black bars identified samples from dairy C whereas dark-gray bars from dairy L. Data are reported as the sum of the intensity of each identified peptide measured as area under the peak (AUP) by Skyline analysis. The complete list of identified peptides can be found in Supplementary Table S1.

peptides came from the hydrolysis of β- and $\alpha_{S1}$-caseins (Fig. 3A). The percentage of incidence of β-casein-derived peptides ranged between 35.12 % and 36.62 % whereas for $\alpha_{S1}$-casein-derived peptides from 32.81 % to 34.58 %. A smaller number of peptides was identified from κ- and $\alpha_{S2}$-caseins (range of incidence 14.29–15.63 % and 12.17–13.09 %, respectively), whereas the lowest number of identified peptides were derived from the hydrolysis of β-lactoglobulin (range 2.86–3.49 %).

The peptide profiles of the *in vitro* digested PR samples from the same dairy were similar, regardless the ripening time. Samples from dairy C shared 85.3 % of identified peptides (348 peptides) and, similarly, samples from dairy L had 83.6 % of peptides in common (346 peptides) (Supplementary Fig. S1). The comparison of the peptide profiles of the samples from the two dairies as a function of ripening time revealed high similarity with 90.8 %, 88.5 %, 91.4 % and 87.9 % of peptides shared between dairies C and L at 12, 18, 24 and 30 months of ripening, respectively (Supplementary Fig. S2).

The total peptide abundance analysis (sum of the area under the peak of each identified peptide) revealed significant differences ($P < 0.05$) in the *in vitro* digested PR samples at 12, 18 and 24 between the two dairies (Fig. 3B). In particular, the *in vitro* digested PR sample at 12 months of ripening from dairy C displayed significantly higher ($P < 0.05$) total peptide abundance compared to the corresponding sample from dairy L, whereas in the PR samples at 18 and 24 months of ripening the total peptide abundance was significantly higher ($P < 0.05$) in samples from dairy L respect to dairy C. Finally, no significant differences ($P > 0.05$) were found in the 30-month ripened PR samples after *in vitro* digestion between diaries C and L (Fig. 3B). When the effect of ripening time was considered, differences were found between dairies C and L. For dairy L, the total peptide abundance increased significantly ($P < 0.05$) from *in vitro* digested PR sample at 12 months to the sample at 18 months of ripening and then remained constant until 30 months of ripening (Fig. 3B). In contrast, for dairy C the total peptide abundance decreased ($P < 0.05$) from *in vitro* digested PR sample at 12 months to the sample at 24 months of ripening and then increased ($P < 0.05$) in the sample at 30 months of ripening.

The highest total peptide abundance *per* protein was found for β-casein in any sample followed by $\alpha_{S1}$-casein, κ-casein, $\alpha_{S2}$-casein, and β-lactoglobulin (Supplementary Fig. S3).

Principal component analysis (PCA) was carried out to explore the possible relationships between dairies and the ripening time. As showed in Fig. 4, when the complete peptide profile was considered, the samples were randomly arranged in space although the two main components describe more than 85 % of the data. This data distribution is interesting because, as reported by Martini et al. (2024), before *in vitro* digestion, PR samples of the same ripening time tended to be arranged close together in space, whereas after *in vitro* digestion, the spatial arrangement of samples was random, being heterogeneously distributed along the principal components A similar behavior before and after *in vitro* digestion of PR cheese was also observed by Bottari et al. (2020) and by Castellone et al. (2022). Indeed, Bottari et al. (2020) reported that samples of PR cheese (from curd to 24 months of ripening) from different dairies were spatially distributed according to different ripening times. This suggests that the ripening process leads to the formation of similar peptides that can be considered as markers for certain ripening times. In contrast, by analyzing samples at the same ripening time after *in vitro* digestion, Castellone et al. (2022), observed that the samples no longer clustered by ripening time but rather randomly. These results highlighted that *in vitro* digestion had a deeper impact on the peptide profile compared to ripening time or production dairy.

### 3.4. Identification of peptides with previously reported biological activities

By using the Milk Bioactive Peptides Database, a total of 64 bioactive peptides were identified considering all the *in vitro* digested PR samples (Supplementary Table S2). Out of the 64 bioactive peptides identified, 34 were derived from β-casein hydrolysis, 15 from $\alpha_{S1}$-casein hydrolysis, 7 from κ-casein hydrolysis, 5 from β-lactoglobulin hydrolysis and 3 from $\alpha_{S2}$-casein hydrolysis. Regarding the bioactivity, 32 peptides were ACE-inhibitors, 18 were antioxidant and 8 were DPP-IV-inhibitors. Additional detected bioactivities were anti-microbial (15 peptides), opioid (6 peptides), immunomodulatory (5 peptides), and anti-cancer (4 peptides). Furthermore, 4 poly-phosphorylated peptides were able to increase the calcium uptake. Among the 64 identified bioactive peptides, 23 were multifunctional exhibiting at least two different bioactivities. Most bioactive peptides (59 out of 64) were identified in all the samples. The lowest frequencies of identification were found for the ACE-inhibitory peptides NMAINPSK which was detected in 4 out of 8 PR samples and LPLP identified in 6 samples.

The total bioactive peptide abundance followed a similar increasing trend as a function of the ripening time for both the

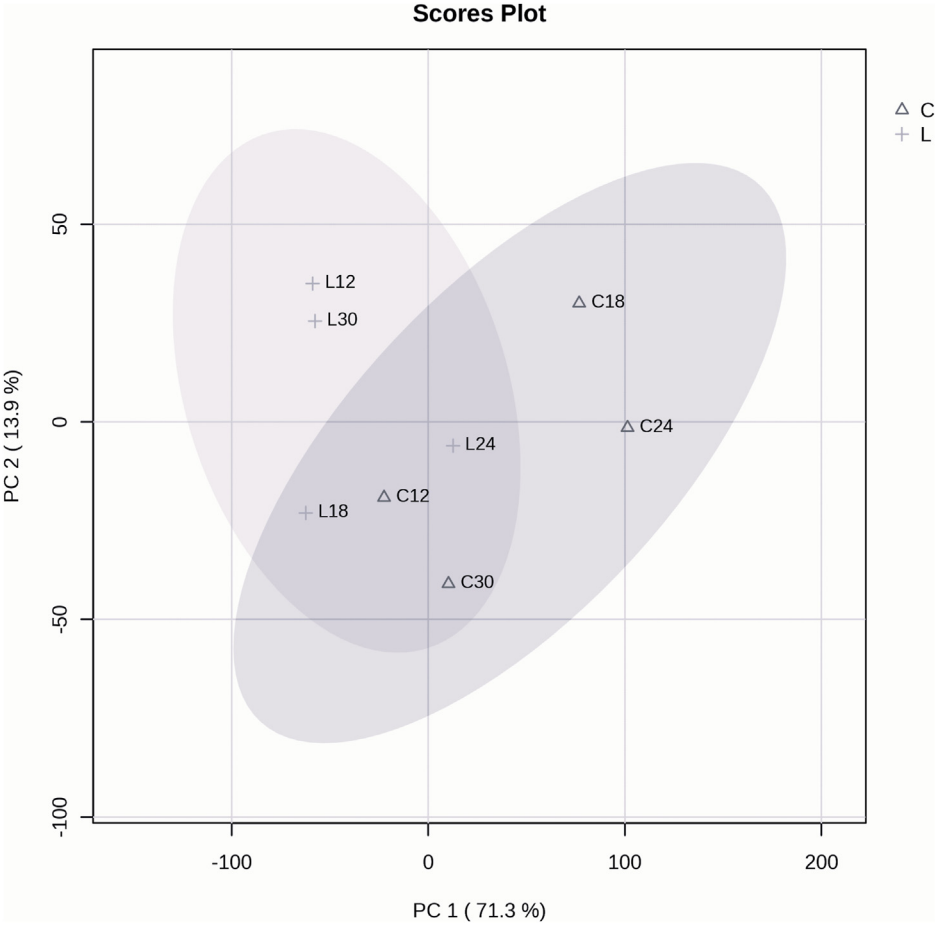


**Fig. 4.** Chemometric analysis (score plot) of the total peptide profile of *in vitro* digested Parmigiano Reggiano samples from dairy C and dairy L at different ripening times (12, 18, 24 and 30 months of ripening).

dairies without any evident differences between dairies, except for the *in vitro* digested PR sample at 24 months of ripening (Fig. 5A). This is in agreement with previous studies (Castellone et al., 2022; Martini et al., 2020).

Dairies C and L did not differ in total peptide abundance of ACE-inhibitory peptides ($P > 0.05$) at the same ripening times except for the *in vitro* digested PR sample at 24 months of ripening (Fig. 5B). No clear relationship was observed between the total peptide abundance of ACE-inhibitory peptides and the $IC_{50}$ values (Figs. 5B and 2B). *In vitro* digested samples from dairy C did not differ in total peptide abundance of antioxidant peptides over ripening time ($P > 0.05$) until 24 months of ripening. At 30 months of ripening total peptide abundance of antioxidant peptides reached the highest value (Fig. 5C). Differently, in dairy L, the total peptide abundance of antioxidant peptides continuously increased as a function of ripening reaching the highest value in *in vitro* digested PR sample at 24 months of ripening (Fig. 5C). The same behavior was recorded for the total peptide abundance of DPP-IV-inhibitory peptides (Fig. 5D). Once again, no clear relationship was observed between the total peptide abundance of antioxidant and DPP-IV-inhibitory peptides and the ABTS and $IC_{50}$ values (Figs. 5C, D, 2A and 2D).

Among the identified ACE-inhibitory peptides, 7 had previously reported *in vivo* anti-hypertensive activity in spontaneously hypertensive rats (SHR). The β-casein-derived peptide KVLPVPQ, originally isolated from a casein hydrolyzate, was able to decrease systolic blood pressure by 31.5 mmHg (4.20 kPa) in SHR when administered at doses of 2 mg $kg^{-1}$ (Maeno, Yamamoto, & Takano, 1996). Another β-casein-derived peptide, LHLPLP, displayed potent *in vivo* anti-hypertensive activity in SHR (−25.3 mmHg; −3.37 kPa) at doses of 3 mg $kg^{-1}$ and was already found in several *in vitro* digested cheeses including Parmigiano Reggiano, Grana Padano, and Ras cheese (Basiricò et al., 2015; Helal et al., 2023a; Martini et al., 2020; Miguel, Recio, Ramos, Delgado, & Aleixandre, 2006; Stuknyte et al., 2015). The shorter peptide HLPLP, derived from the hydrolysis of LHLPLP still retained high *in vivo* anti-hypertensive activity (−23.5 mmHg; −3.13 kPa) in SHR (Miguel, Gómez-Ruiz, Recio, & Aleixandre, 2010). This peptide has been already identified after *in vitro* digestion of different cheeses such as Grana Padano, Parmigiano Reggiano, Ras, Cheddar, and Gorgonzola (Basiricò et al., 2015; Helal et al., 2023a; Martini et al., 2020; Stuknyte et al., 2015). Furthermore, the derived fragment LPLP displayed *in vivo* anti-hypertensive activity in SHR although with less efficacy than the two longer peptides (−16 mmHg; −2.13 kPa) (Sánchez-Rivera et al., 2016). Two additional β-casein-derived peptides, AVPYPQR and YPFPGPIPN, were able to decrease systolic blood pressure in SHR but with low potency and a high dosage (Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000; Tagliazucchi et al., 2019). Finally, one $\alpha_{S1}$-casein-derived peptide (AYFYPEL) was characterized as a potent anti-hypertensive peptide able to decrease the systolic blood pressure by 20 mmHg (2.67 kPa) in SHR (Contreras, Carrón, Montero, Ramos, & Recio, 2009). Once again, this peptide has been previously detected in digested cheeses including Parmigiano Reggiano, Cheddar, Gorgonzola, Caprino, Ras and Maasdam (Basiricò et al., 2015; Helal et al., 2023a; Martini et al., 2020; Stuknyte et al., 2015).

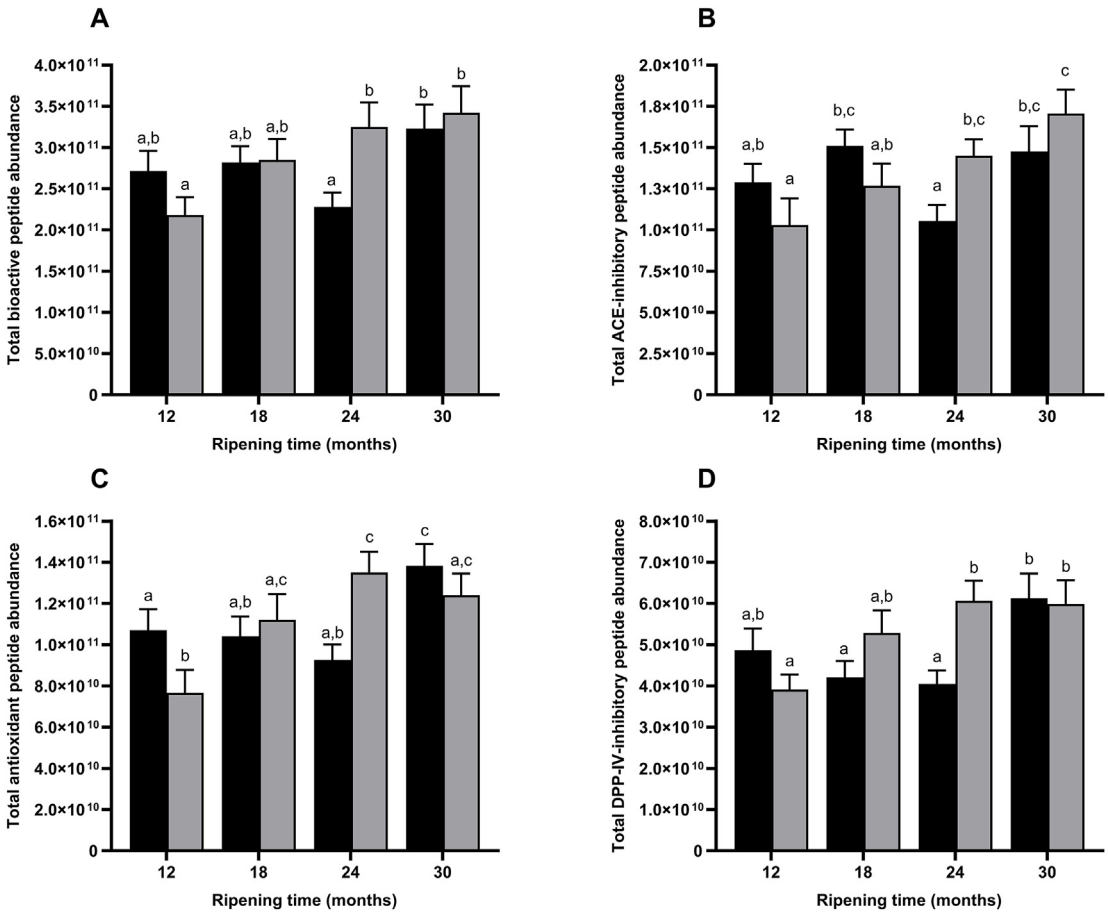


**Fig. 5.** Bioactive peptide abundance in *in vitro* digested Parmigiano Reggiano cheese samples. Analysis was performed on the low molecular weight peptide fractions (<3 kDa) obtained from the *in vitro* digested Parmigiano Reggiano cheese samples. (A) Total bioactive peptide abundance. (B) Total antioxidant peptide abundance. (C) Total ACE-inhibitory peptides abundance. (D) Total DPP-IV-inhibitory peptide abundance. Black bars identified samples from dairy C whereas dark-gray bars from dairy L. Data are reported as the sum of the intensity of each identified peptide measured as area under the peak (AUP) by Skyline analysis. The complete list of identified bioactive peptides can be found in Supplementary Table S2.

Regarding the antioxidant peptides, 12 out of 18 identified antioxidant peptides contained in their sequence the amino acid Y whose presence in the peptide sequence is considered pivotal in determining the antioxidant potential of a peptide (Tagliazucchi, Helal, Verzelloni, & Conte, 2016).

Among the DPP-IV-inhibitory peptides, three of them, namely LPVPQ, IPIQY, and INNQFLPYPY, exhibited low $IC_{50}$ values and have been already identified in *in vitro* digested Parmigiano Reggiano and Ras cheeses (Helal et al., 2023a; Martini et al., 2020).

A major concern about the supposed effect of bioactive peptides on human health is related to their bioavailability (Foltz, van der Pijl, & Duchateau, 2010; Miner-Williams, Stevens, & Moughan, 2014). To this purpose, 3 peptides with reported *in vivo* anti-hypertensive activity in SHR (AYFYPEL, AVPYPQR and YPFPGPIPN) identified in the present study in *in vitro* digested PR samples were detected in human plasma after consumption of milk, suggesting that they are bioavailable in humans (Caira et al., 2022). Furthermore, a total of 7 antioxidant peptides (AYFYPEL, NVPGEIVESL, VYPFPGPIPN, YPFPGPIPN, VLPVPQK, AVPYPQR, and YQEPVLGPVR) were found to be bioavailable in human volunteers after milk consumption pointing out their possible antioxidant effect *in vivo* (Caira et al., 2022). Despite it all, antioxidant peptides may exert their effect in the gastro-intestinal tract independently of their bioavailability as previously suggested (Tagliazucchi et al., 2016). The majority of the identified antioxidant peptides (11 out of 18) have been previously identified and detected *in vivo* in the gastro-intestinal tract of humans or animals (Boutrou et al., 2013; Boutrou, Henry, & Sanchez-Rivera, 2015). DPP-IV inhibitory peptides exert their activity at the gastro-intestinal level (Mulvihill & Drucker, 2014). Three DPP-IV-inhibitory peptides (LPVPQ, YPFPGPIPN and YPVEPF) have been previously identified in the gastro-intestinal tract of healthy human volunteers suggesting their possible effect *in vivo* (Boutrou et al., 2013; Boutrou et al., 2015).

Subsequently, the bioactive peptide abundances and the various bioactivities were correlated (Fig. 6). By looking at the biplot in Fig. 6, vectors indicated that the ACE-inhibitory activity was highly correlated with several peptides, among which we found five known ACE-inhibitory peptides including three with potent ACE-inhibitory activity (low $IC_{50}$) and one with medium ACE-inhibitory activity. These peptides were: AYFYPEL (8), YFYPEL (10), AVPYPQ (47), LLF (62), and LYQEPVLGPV (50) (with $IC_{50}$ of 6.8, 8.8, 15 and 80 μmol $L^{-1}$, respectively). Furthermore, samples from dairy C at 24 and 30 months of ripening were positively correlated with ACE-inhibitory activity, whereas dairy L samples at 12, 18 and 24 months of ripening were in the opposite quadrant (Fig. 6). In fact, the most ripened samples from dairy C showed the highest activity against the ACE enzyme while samples at shorter ripening times (especially samples L at 12 and 18 months of ripening) displayed the lowest inhibitory activity. A similar behavior was already reported by Martini et al. (2020) who found that the amount of ACE-inhibitory peptides detected after *in vitro* digestion of PR cheese increased according to the ripening time.

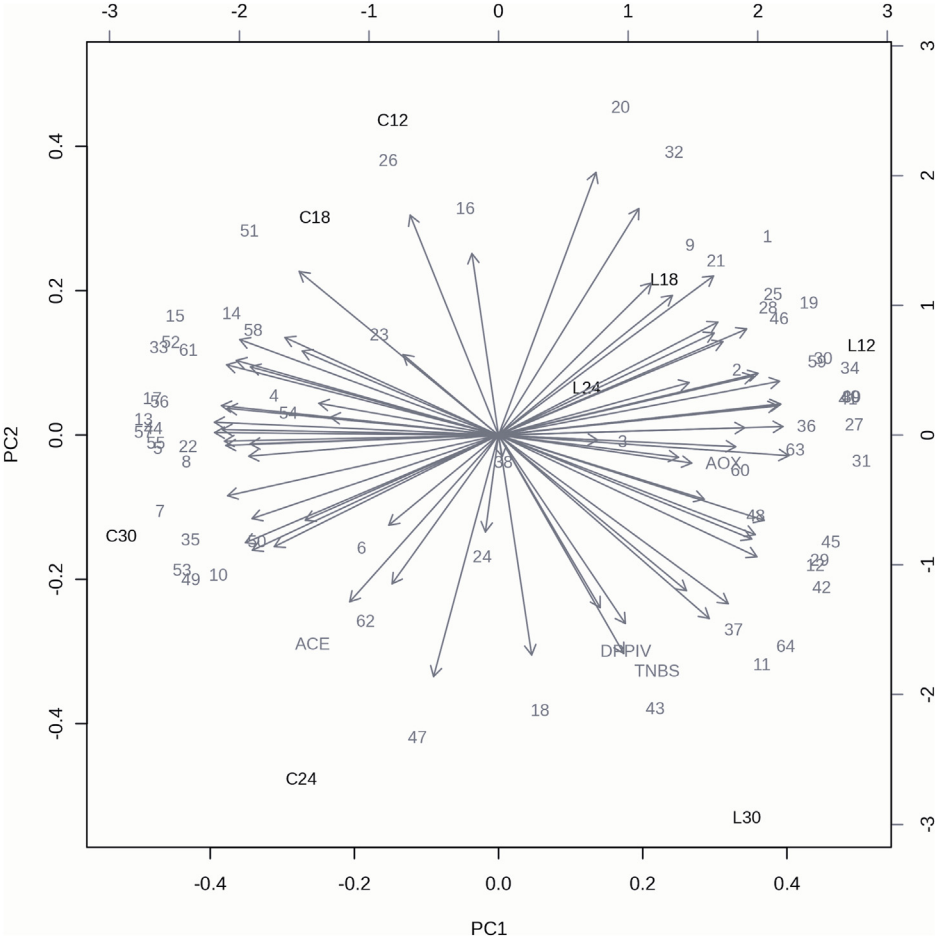


**Fig. 6.** PCA biplot of bioactive peptide data and bioactivities of Parmigiano Reggiano digested samples at different ripening times (12, 18, 24 and 30 months) from two different dairies (dairy C and dairy L). AOX: antioxidant activity; ACE: ACE-inhibitory activity; DPPIV: DPP-IV-inhibitory activity; TNBS: degree of hydrolysis. The numbers identified specific bioactive peptides as reported in Supplementary Table S2.

Furthermore, the DPP-IV-inhibitory activity was found to be positively correlated with TNBS, which suggests that the greater the protein hydrolysis in the sample the greater the inhibitory activity toward the DPP-IV enzyme. In addition, the sample from dairy L at 30 months of ripening was the closest to TNBS and DPP-IV inhibitory activity compared to the other samples. In fact, the sample from dairy L at 30 months of ripening showed highest protein hydrolysis and the lowest $IC_{50}$ towards DPP-IV. Finally, peptide YPVEPF (37), a known DPP-IV inhibitory peptide with a low $IC_{50}$ (150 μmol $L^{-1}$ M) was positively correlated with the DPP-IV inhibitory activity.

Regarding antioxidant activity, this was positively correlated with three antioxidant peptides. These peptides were PEL (12), YPFPGPI (27), PYPQ (48). In addition, antioxidant activity was also positively correlated with the peptide LPYPY (59), which might have antioxidant activity due to the presence of the amino acid tyrosine (Y) in its sequence. Finally, 12- and 24-month-ripened samples from dairy L were positively correlated with the antioxidant activity and also showed the highest values in the antioxidant activity assay.

## 4. Conclusion

The present study highlights the relationships between ripening, *in vitro* digestion, and the biological activity profiles of PR cheese. Peptidomics analysis on the entire pool of peptides after *in vitro* digestion revealed that gastro-intestinal hydrolysis had a greater impact on the peptide profile of the digested PR samples than ripening time. However, ripening had an evident influence on protein hydrolysis, biological activity, and bioactive peptide profile of *in vitro* digested PR samples. Protein hydrolysis after *in vitro* gastro-intestinal digestion of PR samples increased according to the ripening time. The samples at the highest ripening time showed a higher ACE-inhibitory and DPP-IV-inhibitory activity after *in vitro* digestion. Chemometrics analysis revealed that the highest ACE-inhibitory activity of *in vitro* digested PR samples at 24 and 30 months of ripening was positively correlated with the relative amount of several ACE-inhibitory peptides that exhibited very low $IC_{50}$ values, whereas the highest DPP-IV-inhibitory activity of *in vitro* digested long-ripened PR samples was positively correlated with the data from the TNBS assay which is a measure of protein hydrolysis.

Therefore, even if the hydrolysis carried out by gastro-intestinal proteases determined a uniformity in the total peptide profile of PR samples, the digested PR cheeses at the different ripening times displayed different *in vitro* biological activities and bioactive peptide profiles. Consequently, ripening time may have a deep impact on the bio-functionalities of PR cheese and may differently affect human health. However, further studies are necessary to verify the *in vivo* impact of PR consumption on human health and the physiological role of bioactive peptides.

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## CRediT authorship contribution statement

**Alice Cattivelli:** Writing – review & editing, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Lisa Solieri:** Writing – review & editing, Validation, Methodology, Funding acquisition, Data curation, Conceptualization. **Serena Martini:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Data curation, Conceptualization. **Valentina Pizzamiglio:** Writing – review & editing, Validation, Methodology, Data curation, Conceptualization. **Davide Tagliazucchi:** Writing – original draft, Validation, Supervision, Project administration, Methodology, Funding acquisition, Data curation, Conceptualization.

## 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.

## Data availability

Data will be made available on request.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.idairyj.2024.106028.

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