Effect of ripening and *in vitro* digestion on the evolution and fate of bioactive peptides in Parmigiano-Reggiano cheese

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1 **ABSTRACT**

2 The influence of ripening (12, 18 and 24 months) and *in vitro* digestion on the peptidomic profile of

3 Parmigiano-Reggiano (PR) cheeses were investigated. Ripening and i*n vitro* digestion thoroughly

4 modified the peptidomic profile of the three cheeses. Twenty-six bioactive peptides were identified

5 in undigested PR. Some peptides were degraded and others released during ripening. After

6 digestion, 52 bioactive peptides were identified. Semi-quantitative data suggested that bioactive

7 peptides released after digestion can be clustered in 5 groups according to the ripening time. VPP

8 and IPP peptide levels in undigested samples were in the range of 4.52-11.34 and 0.66-4.24 mg Kg^{-}

9 ^{1}, with the highest amounts found in 18-month ripened PR. YPFPGPI peptide was absent in

10 undigested PRs but was released after digestion, especially in the 12-month-old sample (20.18 mg

11 Kg^{-1}). The present study suggests possible differences in bioactive peptide levels after digestion as a

12 function of the duration of ripening of PR cheese.

1. Introduction

Cheese ripening is characterized by a complex chain of events that entails an intricate set of biochemical reactions. Among the different biochemical events occurring during cheese ripening, proteolysis is undeniably one of the most important. Several enzymatic activities originating from various sources are involved in the proteolysis process during cheese ripening. Curd and endogenous milk proteolytic enzymes (such as plasmin) initially hydrolyze caseins, generating large or intermediate-size peptides. The released peptides are further cleaved by the action of proteinases and peptidases coming from starter (S-LAB) and nonstarter (NS-LAB) lactic acid bacteria (Sforza et al., 2012).

Bioactive peptides can be defined as short amino acid sequences, originally encrypted within the sequence of the parent protein, which can be released after proteolysis and may have a positive impact on human health (Rizzello et al., 2016). Numerous bioactive peptides have been identified and characterized after hydrolysis of different food proteins or in fermented dairy products, presenting different functional activities including antimicrobial, antioxidative, dipeptidyl peptidase-IV (DPP-IV) and angiotensin-converting enzyme (ACE) inhibition, antihypertensive, immunomodulatory and opioid activities (Nongonierma & FitzGerald, 2015; Rizzello et al., 2016). In cheese, the presence of bioactive peptides is the result of a sensitive equilibrium between their release and their degradation by the activity of lactic acid bacteria proteinases and peptidases during cheese ripening (Sforza et al., 2004; Sforza et al., 2012). Numerous bioactive peptides, especially ACE-inhibitory and anti-hypertensive peptides, have been identified in various cheeses (Sieber et al., 2010; Lu, Govindasamy-Lucey, & Lucey, 2015; Stuknyte, Cattaneo, Masotti, & De Noni, 2015; Basiricò et al., 2015). Meyer, Bütikofer, Walther, Wechsler, & Sieber (2009) investigated the changes in concentration of the lactotripeptides VPP and IPP in different Swiss cheese varieties during the ripening; they found that the concentration of VPP and IPP increased in semi-hard cheeses according to the ripening time whereas, in hard cheeses, the behavior was dependent on the cheese varieties. Gómez-Ruiz, Ramos, & Recio (2004) and Ong, & Shah (2008) investigated the

release of 5 and 6 ACE-inhibitory peptides during ripening of Manchego and Cheddar cheeses,
respectively. In both cases, the authors did not find a general trend for the release of those peptides
during cheese ripening.

Parmigiano-Reggiano is a long ripened, hard cheese made from raw cow milk and whey starter as
sources of fermenting microorganisms (Solieri, Bianchi, & Giudici, 2012). Parmigiano-Reggiano is
characterized by positive nutritional qualities, being an important source of essential nutrients, such
as proteins, fat, vitamins and minerals, and is considered a functional food due to the presence of
different compounds with particular biological activities (Summer et al., 2017; Godos et al., 2019).
Very few studies have investigated the presence of bioactive peptides and their fate during ripening
in Parmigiano-Reggiano. Sforza et al. (2012) gave the most detailed scenario of the evolution of
peptides during Parmigiano-Reggiano ripening. Several bioactive peptides were found to be present
in Parmigiano-Reggiano, such as the antimicrobial peptide isracidin (α_{S1}-casein fragment 1-23), the
multifunctional bioactive peptides YQEPVLGPVRGPFPIIV (β-casein fragment 193-209) and some
caseinophosphopeptides (Sforza et al., 2012). Basiricò et al. (2015) identified and quantified 4 anti-
hypertensive peptides (VPP, IPP, HLPLP and LHLPLP) in the water-soluble extract of 12-months
ripened Parmigiano-Reggiano. However, a detailed picture of the presence and evolution of such
bioactive peptides during cheese ripening is still lacking.

In addition, bioactive peptides might be degraded by gastro-intestinal proteases after ingestion.
Nevertheless, new sequences could be released from inactive or less active precursors after
digestion. Stuknite et al. (2015) identified and quantified 8 ACE-inhibitory peptides in different
types of cheeses, which amounts were variably influenced by *in vitro* gastro-intestinal digestion. In
another study, Sánchez-Rivera et al. (2014) investigated the influence of *in vitro* gastro-intestinal
digestion on the peptidomic profile of Spanish blue cheese. They found that some peptides were
degraded during cheese digestion, whereas some others were newly released by gastro-intestinal
proteases. In this way, at the end of the digestion, a higher number of bioactive peptides were
found. Basiricò et al. (2015) studied the fate of 8 anti-hypertensive peptides during *in vitro* gastro-

65 intestinal digestion of Parmigiano-Reggiano. The concentration of some peptides such as VPP and

66 IPP was mostly un-affected by the *in vitro* digestion, whereas HLPLP and LHLPLP levels greatly

67 increased after the digestive process. Some other peptides, such as AYFYPE and AYFYPEL, were

68 not found in the Parmigiano-Reggiano samples but they were released during *in vitro* digestion.

69 These results suggest that *in vitro* digestion greatly influences the peptidomic profile of cheese.

70 The present study was designed to compare the peptidomic profile of Parmigiano-Reggiano cheese

71 at different times of ripening as well as the influence of *in vitro* gastro-intestinal digestion.

72 Bioactive peptides were identified, relatively quantified (by integration of the peak area of

73 individual peptides) and their fate was followed during ripening and *in vitro* digestion. Finally, three

74 well-known bioactive peptides, namely VPP, IPP and YPFPGPI, were quantified in the different

75 samples before and after *in vitro* digestion.

76

77 **2. Materials and methods**

78 *2.1. Materials*

79 All MS/MS reagents were from Bio-Rad (Hercules, CA, U.S.A.), whereas the chemicals and

80 enzymes for the digestion procedure and hydrolysis degree determination were purchased from

81 Sigma-Aldrich (Milan, Italy). Amicon Ultra-4 regenerated cellulose filters with a molecular weight

82 cut-off of 3 kDa were supplied by Millipore (Milan, Italy). Parmigiano-Reggiano cheese samples at

83 12 (PR12), 18 (PR18) and 24 (PR24) months of ripening were withdrawn from the same cheese

84 factory in the province of Reggio Emilia (Italy). Three different Parmigiano-Reggiano cheese

85 samples for each time-point were analysed; these samples were collected the same day from

86 different batches. Cheese production and ripening were carried out according to the Production

87 Specification regulated by the Safeguarding Consortium. The analysed cheese sample were

88 registered as a Protected Designation of Origin (PDO) cheese. A detailed description of the

89 Parmigiano-Reggiano cheese production and maturation according to the PDO can be found in

90   Sforza et al. (2012). VPP, IPP and YPFPGPI (95% purity) were synthesized by Bio-Fab research

91   (Rome, Italy). All the other reagents were from Carlo Erba (Milan, Italy).

92

93   *2.2. Preparation of water-soluble peptides extract (WSPE) from Parmigiano-Reggiano samples*

94   Water-soluble peptides extracts were obtained as described by Sforza et al. (2012) with slight

95   modifications. Five grams of cheese samples were mixed with 45 mL of 0.1 mmol L^{-1} HCl and

96   homogenized for 1 min (3 cycles) using an Ultra-Turrax homogenizer. The samples were then

97   centrifuged at 4000g for 40 min at 4°C. At the end of the centrifugation, the supernatants were

98   collected and filtered through Whatman filters paper 4 (Maidstone, Kent, UK).

99

100   *2.3. In vitro gastro-intestinal digestion of Parmigiano-Reggiano samples using the harmonized*

101   *protocol*

102   The *in vitro* digestion of Parmigiano-Reggiano samples was carried out by following the protocol

103   previously developed within the COST Action INFOGEST (Minekus et al., 2014). Simulated

104   salivary, gastric and intestinal fluids (SSF, SGF and SIF) were prepared exactly as described by

105   Minekus et al. (2014). Cheese samples (5 g) were mixed with 5 mL of SSF (containing 150 U mL^{-1}

106   of salivary α-amylase), ground and incubated for 5 min at 37°C to reproduce mastication. The bolus

107   was then mixed with 10 mL of SGF (containing 4000 U mL^{-1} of porcine pepsin) and the pH

108   adjusted to 2.0 with 6 mol L^{-1} of HCl. After 2 h of incubation at 37°C, the final intestinal step was

109   carried out by adding 20 mL of SIF (containing pancreatin 200 U mL^{-1} based on trypsin activity).

110   Then, the pH was adjusted to 7.0 and the samples were further incubated at 37°C for 2 h. All

111   samples were immediately cooled on ice, centrifuged at 10000 g for 20 min at 4°C and frozen at -

112   80°C for further analysis. The digestions were performed in triplicate. In addition, a control

113   digestion, which included only the gastro-intestinal juices and enzymes, and water in place of

114   cheese, was carried out to consider the possible impact of the digestive enzymes in the subsequent

analysis. For each digestion, aliquots were taken at the end of the gastric and intestinal phases of digestion.

2.4. Preparation of the peptide fractions and determination of peptides concentration

Low molecular weight peptides from WSPE and digested samples were extracted by ultrafiltration (cut-off 3 kDa) as described in Tagliazucchi et al. (2017). The peptide content in these peptide fractions was determined by measuring the amount of released amino groups using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay and leucine as standard (Adler-Nissen, 1979). The obtained raw data from the digested samples were corrected by the contribution of the control digestion. Data are expressed as mmol leucine equivalent g^{-1} of cheese.

2.5. Identification of low molecular weight peptides by ultra high performance liquid chromatography/high resolution mass spectrometry (UHPLC/HR-MS)

The peptide fractions from WSPE and digested samples were subjected to UHPLC/HR-MS analysis for peptide identification. UHPLC/MS and tandem MS experiments were carried out on an UHPLC Ultimate 3000 separation module interfaced with a Q Exactive Hybrid Quadrupole-Orbitrap Mass Spectrometer (Thermo Scientific, San Jose, CA, USA) using a C18 column (Zorbax SB-C18 reversed-phase, 2.1×50 mm, 1.8 μm particle size, Agilent Technologies, Santa Clara, CA, USA). The mobile phase consisted of (A) H_2O/formic acid (99.9:0.1, v /v) and (B) acetonitrile. The sample (10 μL, 100-fold diluted) was loaded into the column at a flow rate of 0.3 mL/min. The gradient started at 2% B, and grew to 3% B in 2 min. The mobile phase composition was raised to 27% B in 19 min and then to 90% in 4 min. The mass spectrometer was set as follow: spray voltage 3.5 kV, capillary temperature 320°C, sheath gas 40 and auxiliary gas 30. Full MS parameters were: resolution 70000, AGC target 3e6, maximum IT 333 ms and scan range 200 to 2000 *m/z*. MS/MS parameters were: resolution 17500, AGC target 1e5, maximum IT 120 ms and isolation window 3 *m/z*.

The MS/MS spectra were then converted to .mgf files and the peptides were identified by using the Swiss-Prot database through MASCOT (Matrix Science, Boston, MA, USA) protein identification software. The following parameters were considered: enzyme, none; peptide mass tolerance, ± 5 ppm; fragment mass tolerance, ± 0.12 Da; variable modification, oxidation (M) and phosphorylation (ST); maximal number of post-translational modifications permitted in a single peptide, 4. The assignment process was validated by the manual inspection of MS/MS spectra.

2.6. Identification of bioactive peptides

Peptides identified in the peptide fractions from WSPE and digested samples were investigated in relation to bioactive peptides previously identified in the literature using the Milk Bioactive Peptides Database (MBPDB) (Nielsen, Beverly, Qu, & Dallas, 2017). Only peptides with 100% homology to acknowledged functional peptides were considered as bioactive peptides. The relative amount of the bioactive peptides was estimated by integrating the area under the peak (AUP). AUP was measured from the extracted ion chromatograms (EIC) obtained for each peptide (tolerance ± 5 ppm). Data are expressed as AUP g^{-1} of cheese.

2.7. Quantification of VPP, IPP and YPFPGPI by parallel reaction monitoring (PRM)

Synthetic peptides were dissolved in solvent A (H$_2$O/formic acid; 99.9:0.1, v /v) at a concentration of 5 mg mL^{-1}. The selected analytes were quantified by standard addition method spiking known amounts of standard solutions directly to the analyzed samples. For each sample, linear range for VPP and IPP standards were generated by using 0, 4, 8, 16 and 32 µg L^{-1} of standard (final concentrations in the samples). For YPFPGPI, the linear range was obtained at 0, 5, 15, 30, 50 µg L^{-1} of standard (final concentrations in the samples).

The samples (10 µL; 100-fold diluted) were then injected in the same UHPLC/HR-MS instrument as describe above. Each sample was analyzed two times. Mobile phase A was 0.1% formic acid in water and mobile phase B was acetonitrile. The elution gradient started with 2% B, was maintained

for 2 min, and then increased to 15% B between 2 and 6 min. The mobile phase composition was

then increased to 27% B in 15 min and further raised to 90% B in 4 min. The flow rate was set at

0.4 mL min^{-1}.

Ion source parameters was as follow: spray voltage 4 kV, capillary temperature 320 °C, sheath gas

50 and auxiliary gas 25. PRM parameters were as follow: resolution 17500, AGC target 5e5, max

IT 150 ms, MSX count 1 and isolation window 3.0 m/z.

The precursor ions selected for VPP, IPP and YPFPGPI were [M + H] $^{+}$ *m/z* 312.1918, 326.2074

and 790.4134, respectively. The product ion y_2 $^{+}$ at *m/z* 213.1234 was selected for quantitation of

VPP and IPP. The product ion y_2 $^{+}$ at *m/z* 229.1547 was selected for quantitation of YPFPGPI.

Peaks were integrated by using the Genesis algorithm function in the Thermo Xcalibur Quantitative

Browser, and 5 ppm mass tolerance was applied for the extraction of target product ions. For each

sample analyzed, three calibration curves were generated by linear regression analysis and the

concentration of each peptide in the sample was calculated by determining the value of the intercept

in the *x*-axis, which represent the initial analyte concentration in the sample.

2.8. Statistical analysis

All data are presented as mean ± standard deviation (SD) for three replicates for each prepared

sample. Univariate analysis of variance (ANOVA) with Tukey post-hoc test was applied using

GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The differences were considered

significant with $P < 0.05$.

3. Results and discussion

3.1. Total peptides quantification in the peptide fractions of Parmigiano-Reggiano (PR) WSPE and digested samples

During cheese ripening, caseins can be hydrolyzed by the activity of proteases and peptidases mainly derived from S-LAB and NS-LAB (Pangallo et al., 2019). Generally, LAB possess a complex proteolytic system, which is able to hydrolyze milk caseins to short peptides and amino acids to fulfill their amino acid requirements (Tagliazucchi, Martini, & Solieri, 2019). LAB cell-envelope proteases (CEPs) break down caseins into protein fragments (mainly oligopeptides of about 5-30 amino acids) (Solieri, De Vero, & Tagliazucchi, 2018). These peptides can be transported into the cell and further hydrolyzed by cytoplasmic peptidases into smaller peptides and amino acids (Tagliazucchi et al., 2019). As shown in Figure 1, ripening affected the extent of proteolysis in PR samples, as determined by the TNBS assay. The amount of water-soluble low molecular weight peptides did not differ between PR12 and PR18 samples whereas a significant increase was observed in PR24 sample ($P < 0.05$).

Previous studies already confirmed an increase in proteolysis as a function of cheese ripening (Bütikofer, Meyer, Sieber, Walther, & Wechsler, 2008; Stuknite et al., 2015). Gaiaschi et al. (2001) reported that the extent of proteolysis in Grana Padano cheese was high in the first 12 months of ripening, reaching a plateau and further increased after 22 months of ripening.

An increase in the level of low molecular weight peptides was observed for PR cheeses at different ripening time-points after gastric digestion (Figure 1). The amount of peptides released from PR24 after gastric digestion was significantly higher ($P < 0.001$) than that released from PR12 and PR18. No significant differences were observed between PR12 and PR18 after gastric digestion ($P > 0.05$). Intestinal digestion brought about an increase in the amount of low molecular weight peptides in all of the samples. Once again, the concentration of peptides detected after the intestinal digestion step of PR24 was significantly higher than that measured in PR12 and PR18 ($P < 0.05$;

213 Fig. 1). No significant differences ($P > 0.05$) were found between peptide concentrations in PR12

214 and PR18.

215

216 *3.2. Effect of ripening on the peptidomic profile of Parmigiano-Reggiano (PR) WSPE peptide*

217 *fractions*

218 Overall, 278 unique peptides were identified in the three PR WSPE peptide fractions at different

219 ripening time-points (Table S1). According to the TNBS assay data, the PR24 sample contained the

220 highest amount of peptides (257 peptides), whereas the amount of peptides identified in PR12 and

221 PR18 samples was similar (84 and 72 peptides, respectively) and lower compared to the number

222 observed in the PR24 sample (Fig. 2A). The majority of the peptides identified in PR12 and PR18

223 samples were from β-casein (63.1% and 58.3%, respectively), whereas the remaining identified

224 peptides were from α_{S1}-casein (Fig. 2A). PR24 sample also contained peptides released from α_{S2}-

225 casein (13.6% of total peptides). The Venn diagram (Fig. 3A) shows that 64 peptides (23% of total

226 peptides) were commonly found in all the PR samples. Five peptides (1.8% of total peptides) were

227 in common between PR12 and PR18, whereas the PR24 sample contained 191 (68.7% of total

228 peptides) unique peptides. Among the 84 peptides identified in PR12, 15 of them (~18% of peptides

229 identified in PR12) were found only in this sample.

230 The peptidomic profile of the three PR samples highlighted the paramount importance of LAB

231 CEPs in the proteolysis of cheese caseins, as indicated by the cleavage sites in the N-terminal

232 region of α_{S1}-casein. Peptidic bonds at the H_8–Q_9, Q_9–G_{10}, Q_{13}–E_{14}, E_{14}–V_{15}, L_{16}–N_{17} and F_{23}–F_{24}

233 positions are well-known cleavage sites for LAB CEPs (Solieri et al., 2018; Jensen, Vogensen, &

234 Ardö, 2009; Hebert et al., 2008). Another trait indicating the action of CEPs in the production of

235 these peptides is that the majority of cleavage sites in β-casein (61.8%, 63.8% and 74.0% in the

236 PR12, PR18 and PR24, respectively) have been previously reported to be typical for several CEPs

237 from *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus delbrueckii*, *Lactobacillus*

238 *paracasei*, *Lactobacillus lactis* and *Lactobacillus helveticus* (Solieri et al., 2018; Hebert et al., 2008;

11

239 Lozo et al., 2011; Juillard et al., 1995; Miyamoto et al., 2015). It is important to emphasize that a

240 complex and dynamic population of LAB, which thoroughly changes during ripening, is

241 characteristic of PR cheeses (Solieri et al., 2012).

242 The action of the extracellular proteinase and intracellular peptidases in Lactobacillus can explain

243 the presence of the high number of unique peptides in PR24. Several studies showed that the LAB

244 population decreases as the ripening of PR proceeds (Solieri et al., 2012; Coppola et al., 1999).

245 LAB cell lysis may release cell envelope-proteases and intracellular peptidases in the matrices,

246 which can then enhance the proteolysis of caseins or caseins peptides. Indeed, the bacterial lysis

247 decreases the number of vital LAB and therefore the amount of peptides translocated into the cells.

248

249 *3.3. Effect of in vitro digestion on the peptidomic profile of digested Parmigiano-Reggiano (PR)*

250 *peptide fractions*

251 UHPLC/HR-MS analysis revealed different peptide profiles for the peptide fractions from the PR

252 samples after *in vitro* gastro-intestinal digestion (Figs. 2B and 3B). In each sample, the majority of

253 the peptides were from β-caseins (47.8, 40.4 and 38.2% of the total identified peptides in digested

254 PR12, PR18 and PR24, respectively) followed by α_{S1}-casein (29.3, 30.9 and 32.6% of the total

255 identified peptides in digested PR12, PR18 and PR24, respectively) (Fig. 2B).

256 The Venn diagram indicated that 158 identified peptides (corresponding to the 33.7% of total

257 identified peptides) co-existed in the three PR peptide fractions after *in vitro* digestion (Fig. 3B).

258 There were 115, 31 and 24 peptides exclusively found in digested PR12, PR18 and PR24,

259 respectively. The highest similarity in peptide profiles was found between digested PR18 and PR24

260 samples, with 258 common peptides.

261 Only 22, 21 and 37 peptides were commonly found in undigested and digested samples from PR12,

262 PR18 and PR24 peptide fractions, respectively (supplementary Table S1). Among these peptides,

263 14 (30.4% of total peptides) were commonly found in each of the undigested and digested samples

264 (supplementary Fig. S1). The peptides RELEEL, ELEEL, DKIHPF, LVYPFP, EMPFPK,

265 SLVYPFPGPIPN, LVYPFPGPIPN, YPFPGPIPN, VLPVPQK and AVPYPQR were from β-casein

266 whereas the peptides FVAPFPE, VAPFPE, EIVPN and YKVPQ were from α_{S1}-casein. It is not

267 surprising that most of these peptides contain a PXP sequence or a proline residue near to the

268 carboxylic end. These peptide structural motifs increase the resistance to gastro-pancreatic proteases

269 action, which do not readily hydrolyze proline-containing peptides (Tagliazucchi, Helal, Verzelloni,

270 Bellesia, & Conte, 2016). Indeed, most of these PXP-containing peptides have been found after *in*

271 *vivo* or *in vitro* gastro-intestinal digestion of milk or milk proteins (Tagliazucchi et al., 2016;

272 Tagliazucchi, Martini, Shamsia, Helal, & Conte, 2018; Boutrou et al., 2013; Boutrou, Henry, &

273 Sanchez-Rivera, 2015). The peptides found only in undigested cheeses were likely degraded during

274 *in vitro* gastro-intestinal digestion.

275

276 *3.4. Effect of ripening on the evolution and fate of bioactive peptides in Parmigiano-Reggiano*

277 *cheeses*

278 Peptides in the undigested PR peptide fractions from WSPE were compared for sequence matches

279 with the milk bioactive peptide database MBPDB (Nielsen et al., 2017). Across the categories, 26

280 peptides in undigested PR samples (Table 1) shared the same sequence (100% of homology) with

281 functional peptides previously reported to have various bioactivities.

282 Among the peptide fractions from undigested WSPE, the PR24 sample contained the highest

283 amount of bioactive peptides (26 peptides) respect to PR18 and PR12 samples (12 and 11 peptides,

284 respectively) (Table 1 and supplementary Figure S2A). The Venn diagram (supplementary Fig.

285 S2A) shows that 11 bioactive peptides (42.3% of total peptides) were commonly found in all the PR

286 samples, whereas 14 bioactive peptides were uniquely present in the PR24 sample (Table 1). Nine

287 of the identified bioactive peptides were angiotensin converting-enzyme (ACE) inhibitors, 5

288 peptides were anti-microbial, 1 was a di-peptidyl-peptidase IV (DPPIV) inhibitor, 1 was anxiolytic,

289 1 was antioxidant and 9 were multifunctional bioactive peptides. Considering also the

290 multifunctional bioactive peptides, 9 were anti-microbial essentially active against pathogenic

291 Gram-negative bacteria such as *Escherichia coli*, *Cronobacter sakazakii* and *Staphylococcus aureus*

292 (Sedaghati, Ezzattpanah, Mashhadi Akbar Boojar, Tajabadi Ebrahimi, & Kobarfard, 2015;

293 Birkemo, O'Sullivan, Ross, & Hill, 2009; Kent et al., 2012). The ability of LAB present in PR

294 cheese to produce anti-microbial peptides from hydrolysis of milk proteins may confer a

295 competitive advantage, thus decreasing the risk of the growth and survival of food-borne pathogens

296 (Settanni, & Moschetti, 2010).

297 Of the 11 commonly identified peptides, the relative abundance of 10 was significantly higher in

298 P24 sample respect to P12 and P18 samples ($P<0.05$). In contrast, the ACE-inhibitory peptide

299 FFVAPFPEVFGK displayed a decreasing trend during ripening with the highest relative abundance

300 found in P12 sample ($P<0.05$). The ACE-inhibitory peptide SKVLPVPQ was not detected in

301 sample P12 but showed an increasing trend during ripening with the highest relative abundance

302 found in sample P24 ($P<0.05$).

303

304 *3.5. Effect of in vitro digestion on the evolution and fate of bioactive peptides in Parmigiano-*

305 *Reggiano cheeses*

306 The bioactive peptide profile varied in the PR samples after *in vitro* gastro-intestinal digestion

307 (Table 2). Globally, 52 peptides with 100% of homology with previously reported functional

308 peptides were identified in digested PR samples (Table 2). The majority (75%) of total identified

309 bioactive peptides (39 peptides) were commonly found in the three digested PR samples (Table 2

310 and supplementary Fig. S2B). Most of the identified bioactive peptides were ACE-inhibitors (17

311 peptides) and multifunctional peptides (16 peptides). The other identified bioactive peptides were

312 anti-microbial (7 peptides), DPPIV-inhibitors (4 peptides), antioxidant (2 peptides), opioid (2

313 peptides), cathepsin B-inhibitors (2 peptides), prolyl-endopeptidase-inhibitor (1 peptide) and

314 immunomodulatory (1 peptide). Three bioactive peptides (LHLPLP, HLPLP and AYFYPEL) were

315 already reported after *in vitro* digestion of PR cheese at 12 months of ripening (Basiricò et al.,

2015), whereas the other bioactive peptides were identified in digested PR cheeses for the first time in this study.

The resulting data from semi-quantitative analysis demonstrated that the majority of identified bioactive peptides were not present at a constant level after digestion with respect to the ripening time, but each peptide showed a characteristic trend. Bioactive peptides identified after *in vitro* digestion can be clustered into 5 different groups as a function of the evolutive trend respect to the ripening time (Table 2).

The first group was represented by bioactive peptides whose release after *in vitro* digestion continuously increased according to the ripening time (Table 2). This group was mainly characterized by the presence of ACE-inhibitory peptides (10 bioactive peptides out of 13). Most of these peptides showed low or very low IC_{50} values against ACE. The peptides LHLPLPL, LHLPLP and YKVPQL have been reported to reduce hypertension in spontaneously hypertensive rats (SHR) (Quirós et al., 2007; Maeno, Yamamoto, & Takano, 1996; Miguel, Recio, Ramos, Delgado, & Aleixandre, 2006). Peptides LHLPLP, YKVPQL and EMPFPK were also found intact in human gastro-intestinal tract (Boutrou et al., 2013).

Peptide LHLPLP was able to resist *in vitro* gastro-intestinal digestion but it was hydrolyzed to HLPLP by cellular peptidases prior to being transported across Caco-2 cells (Quirós et al., 2008; Tagliazucchi et al., 2006). The latter can actually be absorbed by intestinal cells and has been found in human plasma after oral administration (Van Platerink et al., 2006). It has been suggested that the peptide LHLPLP, released after *in vitro* gastro-intestinal digestion of Grana-Padana cheese, may be partially responsible for the blood pressure lowering effect observed *in vivo* after diet enrichment with Grana-Padana cheese (Stuknite et al., 2015; Crippa et al., 2018).

The second group was characterized by bioactive peptides whose release after *in vitro* digestion increased according to the ripening time reaching a plateau after 18 months of ripening (Table 2). This group contained the majority of anti-microbial peptides and some ACE-inhibitory peptides with demonstrated *in vivo* activity on spontaneously hypertensive rats (SHR) and low IC_{50} values.

The peptide AVPYPQR was able to decrease the blood pressure in SHR and behaved as a multifunctional bioactive peptide also showing anti-microbial, anticoagulant and antioxidant activities (Karaki et al., 1990; Tonolo et al., 2018; Tu et al., 2019).

The third group was characterized by bioactive peptides the release of which after *in vitro* digestion increased according to the ripening time reaching a maximum value at 18 months of ripening (Table 2). To this group belonged peptides with different biological activities. The peptide AYFYPEL presented a very low IC_{50} value against ACE and was able to reduce blood pressure in SHR (Contreras, Carrón, Montero, Ramos, & Recio, 2009).

The fourth group was represented by bioactive peptides whose release after *in vitro* digestion decreased according to the ripening time (Table 2). This group was characterized for the presence of the peptide YPFPGPI (also known as β-casomorphin-7) and its precursors. Some ACE-inhibitory peptides were also found in this group but, with the exception of YPFPGPIPN, they displayed higher IC_{50} values.

Finally, the last group contained peptides whose amount after *in vitro* digestion remained constant throughout ripening (Table 2).

3.5. Quantification of YPFPGPI, VPP and IPP in the peptide fractions of WSPE and digested samples of Parmigiano-Reggiano (PR)

Three peptides, namely VPP, IPP and YPFPGPI with documented *in vivo* effect on humans were quantified in undigested and digested PR samples. The tripeptides VPP and IPP received particular consideration since several *in vivo* studies confirmed their antihypertensive effect on SHR and mildly hypertensive patients (Cicero, Fogacci, & Colletti, 2017; Fitzgerald, Murray, & Walsh, 2004). Vice versa, different studies have suggested adverse effects of YPFPGPI (β-casomorphin-7) on human health, including cardiovascular diseases, diabetes and digestive disorders (Asledottir et al., 2018).

367 VPP and IPP have been detected in the WSPE peptide fractions from undigested PR samples at

368 each ripening time (Table 3). The amount of VPP and IPP found in the 12-month ripened PR were

369 6.87 ± 0.68 and 1.63 ± 0.82 mg kg^{-1}, respectively. These data are in accordance with the range

370 reported by Basiricò et al. (2015) in PR sample at 12 months of ripening. The amount of VPP and

371 IPP increased in the sample at 18 months of ripening, reaching a concentration of 11.34 ± 0.21 and

372 4.24 ± 2.85 mg kg^{-1}, respectively. After that, we observed a strong decline in the concentration of

373 VPP and IPP at 24 months of ripening (4.52 ± 0.28 and 0.66 ± 0.05 mg kg^{-1}, respectively).

374 Peptide YPFPGPI, in contrast, was not detected in any undigested PR sample. This is consistent

375 with the report of De Noni, & Cattaneo (2010), who did not observe YPFPGPI in Grana Padano

376 cheese at 10, 17 or 25 months of ripening. These results suggested that LAB proteases and

377 peptidases are not able to release β-casomorphin-7 during cheese ripening.

378 As shown in Table 3, at the end of the *in vitro* gastro-intestinal digestion, the VPP content of PR12

379 and PR18 remained almost unchanged. Moreover, the quantitative analysis mainly showed an

380 increase in the content of IPP in both the samples ($P<0.05$). In contrast, we observed a significant

381 decrease ($P<0.05$) in VPP concentrations after *in vitro* digestion in the PR24 sample, whereas the

382 amount of IPP was unaltered.

383 β-casomorphin-7, which was not present in the cheese WSPE, was released during *in vitro*

384 digestion of PR samples (Table 3). Previous research highlighted the ability of gastro-intestinal

385 proteases to release β-casomorphin-7 during *in vitro* digestion of milk β-casein variant A1

386 (Asledottir et al., 2018). The concentration of YPFPGPI after *in vitro* digestion decreased during

387 ripening, with the highest concentration found in digested PR12 sample.

388

389 **4. Conclusion**

390 According to the data reported in this study, ripening of PR cheese has an important influence in the

391 release of bioactive peptides and the *in vitro* digestion further increased their number in the PR

392 samples. Most of them were found in all of the samples, but in different amounts. Interestingly, they

393   can be clustered accordingly to ripening time and bioactivities. For example, the majority of the

394   bioactive peptides showing an increasing trend after digestion, as a function of the ripening time,

395   were potent ACE-inhibitory peptides. By contrast, most of the identified anti-microbial peptides

396   reached a plateau after 18 months of ripening. Moreover, the opioid peptides β-casomorphin-7 and

397   its precursor displayed a typical behavior with a decreasing trend after *in vitro* digestion as a

398   function of the ripening time. The present study suggests possible differences in the biological

399   effect after ingestion of PR cheese as a function of the ripening time. The major driving force for

400   consumers to choose a cheese with different ripening times is the organoleptic characteristics but,

401   nevertheless, the peptide profile and bioactivities may also change.

Author contributions

SM, AC and DT conceived and designed the study. SM performed the *in vitro* digestion and bioactivity experiments. SM and DT performed the peptidomic experiments and the bioinformatic analysis. DT wrote the manuscript. SM and AC critically revised the manuscript. All the authors read the manuscript and discussed the interpretation of results.

Acknowledgements

Funding

Declarations of interest

None

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Figure captions

Fig. 1. Effect of ripening time and *in vitro* digestion on the peptide concentrations in Parmigiano-Reggiano (PR). The total amount of peptides was expressed as mmol of leucine equivalent per g of cheese. WSPE means water-soluble peptide extracts and represent the peptide fractions obtained after ultrafiltration (< 3 kDa) of water-soluble peptides extracted from un-digested PR samples. PR12: Parmigiano-Reggiano at 12 months of ripening. PR18: Parmigiano-Reggiano at 18 months of ripening. PR24: Parmigiano-Reggiano at 24 months of ripening. Values are means of data from three independent digestions ± standard deviation (SD). Different letters indicate significantly different values ($P < 0.05$)

Fig. 2. Number of unique peptides identified in the Parmigiano-Reggiano (PR) peptide fractions. (A) Number of peptides in un-digested PR peptide fractions at 12 (PR12), 18 (PR18) and 24 (PR24) months of ripening. (B) Number of peptides in *in vitro* digested PR peptide fractions at 12 (PR12), 18 (PR18) and 24 (PR24) months of ripening. The incidence of the different milk proteins on the released peptides is also shown.

Fig. 3. Venn diagrams of peptides obtained from Parmigiano-Reggiano (PR) peptide fractions. (A) Venn diagram created with all the identified peptides in un-digested PR peptide fractions at 12 (WSPE PR12), 18 (WSPE PR18) and 24 (WSPE PR24) months of ripening (see on line supplementary material Tables S1 for the peptide sequences). (B) Venn diagram created with all the identified peptides in digested PR peptide fractions at 12 (D PR12), 18 (D PR18) and 24 (D PR24) months of ripening (see on line supplementary material Tables S1 for the peptide sequences).

Table 1. Relative amount of bioactive peptides identified in water-soluble extract (WSPE) peptide fractions from Parmigiano Reggiano samples at 12 (PR12), 18 (PR18) and 24 (PR24) months of ripening with previously demonstrated bioactivity[a].

Sequence[b]	Fragment	Relative amount in WSPE PR12[c]	Relative amount in WSPE PR18[c]	Relative amount in WSPE PR24[c]
ACE-inhibitory				
DKIHPF	βCN 47-52	$9.02 \times 10^5 \pm 7.75 \times 10^{4a}$	$9.80 \times 10^5 \pm 5.81 \times 10^{4a}$	$1.41 \times 10^6 \pm 7.43 \times 10^{4b}$
LVYPFP	βCN 58-63	$1.11 \times 10^5 \pm 1.04 \times 10^{4a}$	$1.04 \times 10^5 \pm 2.05 \times 10^{4a}$	$4.01 \times 10^5 \pm 9.12 \times 10^{4b}$
SQSKVLPVPQ	βCN 166-175	n.d.	n.d.	$1.84 \times 10^6 \pm 1.05 \times 10^5$
SKVLPVPQ	βCN 168-175	n.d.	$1.01 \times 10^4 \pm 1.63 \times 10^{2a}$	$4.79 \times 10^5 \pm 1.27 \times 10^{5b}$
VLPVPQK[d,e]	βCN 170-176	$2.89 \times 10^5 \pm 2.82 \times 10^{4a}$	$2.44 \times 10^5 \pm 5.50 \times 10^{4a}$	$3.52 \times 10^6 \pm 1.06 \times 10^{5b}$
RDMPIQAF	βCN 183-190	$9.47 \times 10^3 \pm 8.98 \times 10^{2a}$	$1.09 \times 10^4 \pm 6.11 \times 10^{2a}$	$1.01 \times 10^6 \pm 6.77 \times 10^{4b}$
YQEPVLGPVRGPFPIIV[e,f]	βCN 193-209	$1.32 \times 10^4 \pm 5.95 \times 10^{3a}$	$4.18 \times 10^4 \pm 3.18 \times 10^{3b}$	$4.04 \times 10^5 \pm 4.26 \times 10^{4b}$
QEPVLGPVRGPFPIIV	βCN 194-209	n.d.	n.d.	$5.06 \times 10^5 \pm 7.12 \times 10^4$
FALPQYLK	α_{S2}CN 174-181	n.d.	n.d.	$1.66 \times 10^5 \pm 8.02 \times 10^4$
AMKPWIQPK	α_{S2}CN 189-197	n.d.	n.d.	$4.96 \times 10^5 \pm 6.73 \times 10^4$
Anti-hypertensive				
VYPFPGPIPN[g]	βCN 59-68	n.d.	n.d.	$8.99 \times 10^5 \pm 2.44 \times 10^4$
YPFPGPIPN[g]	βCN 60-68	$6.64 \times 10^4 \pm 5.57 \times 10^{3a}$	$3.33 \times 10^4 \pm 4.34 \times 10^{3b}$	$2.49 \times 10^5 \pm 2.32 \times 10^{4c}$
EMPFPK[g]	βCN 108-113	$1.47 \times 10^4 \pm 4.60 \times 10^{2a}$	$1.77 \times 10^4 \pm 1.44 \times 10^{3b}$	$7.06 \times 10^5 \pm 4.75 \times 10^{4c}$
AVPYPQR[g]	βCN 177-183	$1.89 \times 10^4 \pm 3.16 \times 10^{3a}$	$1.66 \times 10^4 \pm 1.12 \times 10^{3a}$	$4.52 \times 10^5 \pm 4.21 \times 10^{4b}$
LLYQEPVLGPVRGPFPIIV[g]	βCN 191-209	n.d.	n.d.	$1.72 \times 10^6 \pm 2.41 \times 10^5$
FFVAPFPEVFGK[g]	α_{S1}CN 23-34	$1.38 \times 10^6 \pm 9.46 \times 10^{4a}$	$1.19 \times 10^6 \pm 5.61 \times 10^{4b}$	$4.42 \times 10^5 \pm 2.47 \times 10^{5c}$
Anti-microbial				
EAMAPK	βCN 100-105	n.d.	n.d.	$2.27 \times 10^5 \pm 1.77 \times 10^5$
VLPVPQKAVPYPQR	βCN 170-183	n.d.	n.d.	$1.50 \times 10^6 \pm 1.31 \times 10^5$
VLNENLLR	α_{S1}CN 15-22	n.d.	n.d.	$1.76 \times 10^5 \pm 1.08 \times 10^5$
HIQKEDVPSERYLGYLEQLLRLK	α_{S1}CN 80-102	n.d.	n.d.	$1.09 \times 10^5 \pm 7.56 \times 10^4$
YLEQLLR	α_{S1}CN 94-101	n.d.	n.d.	$5.57 \times 10^5 \pm 7.73 \times 10^4$
Immunomodulatory				
PGPIPN	βCN 63-68	$2.08 \times 10^5 \pm 1.20 \times 10^{4a}$	$1.21 \times 10^5 \pm 2.18 \times 10^{4b}$	$2.70 \times 10^5 \pm 3.04 \times 10^{4c}$
Antioxidant				
VKEAMAPK[e]	βCN 98-105	n.d.	n.d.	$2.80 \times 10^5 \pm 2.75 \times 10^4$
TQTPVVVPPFLQPE	βCN 78-91	n.d.	n.d.	$5.57 \times 10^4 \pm 1.64 \times 10^4$
DPPIV-inhibitory				

LPVPQ	βCN 171-175	n.d.	n.d.	$2.25 \times 10^5 \pm 1.14 \times 10^5$

Anxiolytic

YLGYLEQLLR	α_{S1}CN 91-101	$6.79 \times 10^5 \pm 4.29 \times 10^{4a}$	$6.64 \times 10^5 \pm 2.65 \times 10^{4a}$	$1.14 \times 10^6 \pm 4.95 \times 10^{3b}$

[a]Abbreviations are: ACE, angiotensin converting enzyme; DPPIV, dipeptidyl peptidase IV; CN, casein.

[b]One code letter was used for amino acid nomenclature. Potential bioactivities were achieved from MBPDB databases (Nielsen et al., 2017). Anti-hypertensive activity was measured on spontaneously anti-hypertensive rats. Multifunctional peptides were labelled with superscript letters: [d]antioxidant activity; [e]antimicrobial activity; [f]immunomodulator; [g]ACE-inhibitory activity.

[c]Relative amount was expressed as the area under the peak (AUP) g[-1] of cheese measured from the extracted ion chromatograms (EIC) obtained for each peptide. N.d. means peptide not detected in the sample. Different superscript letters within the same row indicate that the values are significantly different ($P<0.05$)

Table 2. Relative amount of bioactive peptides identified in digested peptide fractions from Parmigiano Reggiano samples at 12 (PR12), 18 (PR18) and 24 (PR24) months of ripening with previously demonstrated bioactivity[a]

Sequence[b]	Fragment	Relative amount in digested PR12[c]	Relative amount in digested PR18[c]	Relative amount in digested PR24[c]
Peptides with increasing trend according to the ripening time				
LVYPFP[d]	βCN 58-63	$1.80 \times 10^5 \pm 1.15 \times 10^{4a}$	$1.18 \times 10^5 \pm 8.77 \times 10^{4a}$	$7.60 \times 10^5 \pm 4.66 \times 10^{4b}$
NIPPLTQTPVVVPPFLQ[d]	βCN 73-89	$1.18 \times 10^4 \pm 7.45 \times 10^{3a}$	$6.91 \times 10^5 \pm 5.74 \times 10^{4b}$	$9.09 \times 10^5 \pm 1.50 \times 10^{4c}$
EMPFPK[d,e]	βCN 108-113	$7.28 \times 10^6 \pm 1.02 \times 10^{5a}$	$3.04 \times 10^7 \pm 1.98 \times 10^{6b}$	$7.90 \times 10^8 \pm 3.90 \times 10^{7c}$
LHLPLP[d,f]	βCN 133-138	$2.45 \times 10^8 \pm 1.85 \times 10^{7a}$	$4.33 \times 10^8 \pm 3.88 \times 10^{7b}$	$6.83 \times 10^8 \pm 6.22 \times 10^{7c}$
LHLPLPL[d,f]	βCN 133-139	$2.75 \times 10^7 \pm 1.51 \times 10^{6a}$	$6.95 \times 10^7 \pm 6.87 \times 10^{6b}$	$8.63 \times 10^7 \pm 4.92 \times 10^{6c}$
YQEPVL[d]	βCN 193-198	$5.87 \times 10^4 \pm 4.25 \times 10^{4a}$	$4.38 \times 10^6 \pm 5.53 \times 10^{5b}$	$1.98 \times 10^7 \pm 2.40 \times 10^{5c}$
YKVPQL[d,f]	α$_{S1}$CN 104-109	$4.06 \times 10^4 \pm 3.65 \times 10^{4a}$	$6.25 \times 10^7 \pm 7.97 \times 10^{6b}$	$1.38 \times 10^8 \pm 3.32 \times 10^{7c}$
NMAINPSK[d]	α$_{S2}$CN 25-32	$2.05 \times 10^6 \pm 3.38 \times 10^{6a}$	$3.83 \times 10^7 \pm 2.50 \times 10^{6b}$	$6.00 \times 10^7 \pm 3.12 \times 10^{6c}$
SRYPSY[g]	κCN 33-38	n.d.	$4.81 \times 10^6 \pm 5.04 \times 10^{5a}$	$7.55 \times 10^6 \pm 8.60 \times 10^{5b}$
INNQFLPYPY[h]	κCN 51-60	$1.34 \times 10^5 \pm 1.39 \times 10^{4a}$	$1.10 \times 10^8 \pm 1.26 \times 10^{7b}$	$2.48 \times 10^8 \pm 6.84 \times 10^{6c}$
LPYPY[d,h]	κCN 56-60	$3.61 \times 10^5 \pm 5.51 \times 10^{4a}$	$2.19 \times 10^7 \pm 1.72 \times 10^{6b}$	$4.64 \times 10^7 \pm 3.20 \times 10^{6c}$
IPAVF[h]	βLB 78-82	$4.67 \times 10^5 \pm 6.27 \times 10^{4a}$	$1.88 \times 10^6 \pm 2.90 \times 10^{5b}$	$7.95 \times 10^6 \pm 1.29 \times 10^{5c}$
VLDTDYK[d]	βLB 94-100	n.d.	$2.61 \times 10^6 \pm 1.92 \times 10^{5a}$	$5.27 \times 10^6 \pm 1.15 \times 10^{5b}$
Peptides with increasing trend according to the ripening time reaching a plateau at 18 months				
PVVVPPFLQPE[e]	βCN 81-91	n.d.	$1.57 \times 10^7 \pm 1.67 \times 10^{6a}$	$1.32 \times 10^7 \pm 3.94 \times 10^{6a}$
VENLHLPLPLL[d]	βCN 130-140	n.d.	$1.19 \times 10^6 \pm 1.80 \times 10^{5a}$	$1.44 \times 10^6 \pm 2.64 \times 10^{5a}$
VLPVPQK[d]	βCN 170-176	$2.22 \times 10^7 \pm 3.68 \times 10^{6a}$	$1.00 \times 10^9 \pm 1.49 \times 10^{6b}$	$1.08 \times 10^9 \pm 5.94 \times 10^{6b}$
AVPYPQR[d,e,f,i]	β177-183	$3.00 \times 10^5 \pm 1.31 \times 10^{5a}$	$5.48 \times 10^8 \pm 5.31 \times 10^{7b}$	$6.01 \times 10^8 \pm 2.76 \times 10^{7b}$
DAYPSGAW[d]	α$_{S1}$CN 157-164	$7.32 \times 10^5 \pm 1.43 \times 10^{5a}$	$4.61 \times 10^6 \pm 9.28 \times 10^{5b}$	$5.11 \times 10^6 \pm 9.02 \times 10^{5b}$
SDIPNPIGSENSEK[e]	α$_{S1}$CN 180-193	$3.79 \times 10^6 \pm 5.49 \times 10^{5a}$	$2.48 \times 10^8 \pm 3.92 \times 10^{7b}$	$2.69 \times 10^8 \pm 6.55 \times 10^{7b}$
FFSDK[e]	κCN 17-21	$3.65 \times 10^6 \pm 3.09 \times 10^{5a}$	$1.26 \times 10^7 \pm 1.32 \times 10^{6b}$	$1.71 \times 10^7 \pm 2.43 \times 10^{6b}$
YIPIQY[d]	κCN 25-30	$2.59 \times 10^4 \pm 2.49 \times 10^{4a}$	$9.80 \times 10^6 \pm 1.45 \times 10^{5b}$	$1.23 \times 10^7 \pm 2.95 \times 10^{6b}$
GLDIQK[d,l]	βLB 9-14	n.d.	$4.53 \times 10^6 \pm 7.53 \times 10^{5a}$	$3.95 \times 10^6 \pm 3.39 \times 10^{5a}$
DAQSAPLR[e]	βLB 33-40	n.d.	$1.34 \times 10^6 \pm 1.17 \times 10^{5a}$	$1.87 \times 10^6 \pm 2.95 \times 10^{5a}$
IIAEK[d,l]	βLB 71-75	$1.76 \times 10^6 \pm 1.50 \times 10^{5a}$	$5.46 \times 10^6 \pm 6.79 \times 10^{5b}$	$4.61 \times 10^6 \pm 3.52 \times 10^{5b}$
IDALNENK[e]	βLB 84-91	n.d.	$1.96 \times 10^6 \pm 1.53 \times 10^{5a}$	$2.06 \times 10^6 \pm 1.82 \times 10^{5a}$
Peptides with increasing trend according to the ripening time reaching a maximum at 18 months				
TEDELQDKIHPF[e]	βCN 41-52	$1.80 \times 10^5 \pm 1.93 \times 10^{4a}$	$4.39 \times 10^6 \pm 1.81 \times 10^{5b}$	$3.80 \times 10^6 \pm 3.45 \times 10^{4c}$
DKIHPF[d]	βCN 47-52	$7.34 \times 10^5 \pm 5.30 \times 10^{4a}$	$2.66 \times 10^6 \pm 1.77 \times 10^{5b}$	$1.28 \times 10^6 \pm 5.57 \times 10^{4c}$
PGPIPN[m]	βCN 63-67	$7.73 \times 10^6 \pm 2.89 \times 10^{4a}$	$8.89 \times 10^6 \pm 4.46 \times 10^{4b}$	$6.55 \times 10^6 \pm 3.66 \times 10^{4c}$
EAMAPK[e]	βCN 100-105	$1.61 \times 10^8 \pm 2.06 \times 10^{7a}$	$2.55 \times 10^8 \pm 3.22 \times 10^{6b}$	$1.61 \times 10^8 \pm 3.29 \times 10^{7a}$
KVLPVPQK[i]	βCN 169-176	$1.89 \times 10^4 \pm 2.73 \times 10^{3a}$	$5.85 \times 10^6 \pm 2.34 \times 10^{4b}$	$3.35 \times 10^6 \pm 1.88 \times 10^{5c}$

Peptide	Fragment			
QEPVL[m]	βCN 194-198	n.d.	$1.17\times10^7 \pm 2.08\times10^5$	n.d.
AYFYPEL[d,f,g,i]	α_{S1}CN143-149	$2.19\times10^5 \pm 2.51\times10^{4a}$	$5.54\times10^6 \pm 6.29\times10^{5b}$	$1.38\times10^6 \pm 1.67\times10^{5c}$
FYPEL[d,i]	α_{S1}CN145-149	$5.89\times10^8 \pm 5.16\times10^{7a}$	$9.52\times10^8 \pm 3.07\times10^{7b}$	$6.43\times10^8 \pm 6.22\times10^{7a}$
IPIQY[h]	κCN 26-30	n.d.	$1.28\times10^8 \pm 6.83\times10^{6a}$	$6.56\times10^7 \pm 1.23\times10^{7b}$

Peptides with decreasing trend according to the ripening time

Peptide	Fragment			
HKEMPFPK[e]	βCN 106-113	$5.39\times10^6 \pm 4.36\times10^{5a}$	$3.96\times10^6 \pm 2.55\times10^{5b}$	n.d.
TQTPVVVPPFLQPE[i]	βCN 78-91	$1.04\times10^7 \pm 8.33\times10^5$	n.d.	n.d.
LVYPFPGPI[d]	βCN 58-66	$3.32\times10^7 \pm 7.09\times10^{5a}$	$2.20\times10^7 \pm 7.67\times10^{5b}$	$1.55\times10^7 \pm 3.63\times10^{5c}$
VYPFPGPI[n]	βCN 59-66	$4.62\times10^8 \pm 4.34\times10^{6a}$	$3.75\times10^8 \pm 7.67\times10^{6b}$	$2.24\times10^8 \pm 5.46\times10^{6c}$
VYPFPGPIPN[d,i]	βCN 59-68	$5.35\times10^8 \pm 1.86\times10^{7a}$	$4.70\times10^8 \pm 8.66\times10^{6b}$	$3.20\times10^8 \pm 4.84\times10^{6c}$
YPFPGPI[d,g,m]	βCN 60-66	$6.87\times10^6 \pm 1.29\times10^{5a}$	$2.49\times10^6 \pm 1.34\times10^{5b}$	$1.79\times10^6 \pm 5.68\times10^{4c}$
YPFPGPIPN[d,f,h]	βCN 60-67	$1.13\times10^7 \pm 3.80\times10^{5a}$	$3.13\times10^6 \pm 2.88\times10^{5b}$	$2.66\times10^6 \pm 1.02\times10^{5c}$
PFPGPI[o]	βCN 61-66	$9.65\times10^7 \pm 1.02\times10^{6a}$	$4.84\times10^7 \pm 2.01\times10^{6b}$	$3.56\times10^7 \pm 1.36\times10^{6c}$
NIPPLTQTPV[d]	βCN 73-82	$1.45\times10^8 \pm 1.73\times10^6$	n.d.	n.d.
HLPLP[d]	βCN 134-138	$2.27\times10^8 \pm 1.06\times10^7$	n.d.	n.d.
LPVPQ[h]	βCN 171-175	$1.25\times10^6 \pm 1.55\times10^{5a}$	$4.58\times10^5 \pm 9.46\times10^{4b}$	$2.07\times10^5 \pm 1.29\times10^{4c}$
FVAPFPEVFG[d]	α_{S1}CN 24-33	$8.90\times10^6 \pm 1.75\times10^{6a}$	$3.21\times10^5 \pm 2.94\times10^{5b}$	$5.80\times10^5 \pm 4.05\times10^{5c}$
YFYPE[g]	α_{S1}CN 144-148	$3.36\times10^7 \pm 1.13\times10^{6a}$	$1.59\times10^6 \pm 9.18\times10^{5b}$	$2.02\times10^5 \pm 1.95\times10^{5c}$
AMKPW[d]	α_{S2}CN 189-193	$2.15\times10^7 \pm 3.04\times10^{6a}$	$1.32\times10^7 \pm 1.08\times10^{6b}$	$1.58\times10^7 \pm 6.12\times10^{5c}$

Peptides found in constant amount according to the ripening time

Peptide	Fragment			
YPVEPF[g,h]	βCN 114-119	$1.40\times10^9 \pm 7.92\times10^{7ab}$	$1.44\times10^9 \pm 1.98\times10^{7a}$	$1.38\times10^9 \pm 2.99\times10^{7b}$
GPFPI[o]	βCN 203-207	$1.96\times10^9 \pm 4.74\times10^{7a}$	$1.72\times10^9 \pm 9.60\times10^{7a}$	$1.82\times10^9 \pm 3.61\times10^{7a}$
YFYPEL[g,i]	α_{S1}CN 144-149	$3.35\times10^7 \pm 5.18\times10^{6a}$	$4.54\times10^7 \pm 7.41\times10^{6a}$	$3.66\times10^7 \pm 5.20\times10^{6a}$
TPEVDDEALEK[e,h]	βLB 125-135	$1.69\times10^7 \pm 1.93\times10^{6a}$	$2.06\times10^7 \pm 2.43\times10^{6a}$	$1.85\times10^7 \pm 2.97\times10^{6a}$

[a]Abbreviations are: CN, casein; LB, lactoglobulin.

[b]One code letter was used for amino acid nomenclature. Potential bioactivities were achieved from MBPDB databases (Nielsen et al., 2017). Anti-hypertensive activity was measured on spontaneously anti-hypertensive rats. Bioactive peptides are labelled as follow: [d]ACE-inhibitory activity; [e]anti-microbial activity; [f]anti-hypertensive activity; [g]opioid; [h]DPPIV-inhibitory activity; [i]antioxidant activity; [l]hypocholesterolemic; [m]immunomodulator; [n]PEP-inhibitory activity; [o]catepsin B-inhibitory activity. Abbreviations are: ACE, angiotensin converting enzyme; DPPIV, dipeptidyl peptidase IV; PEP, prolyl endopeptidase.
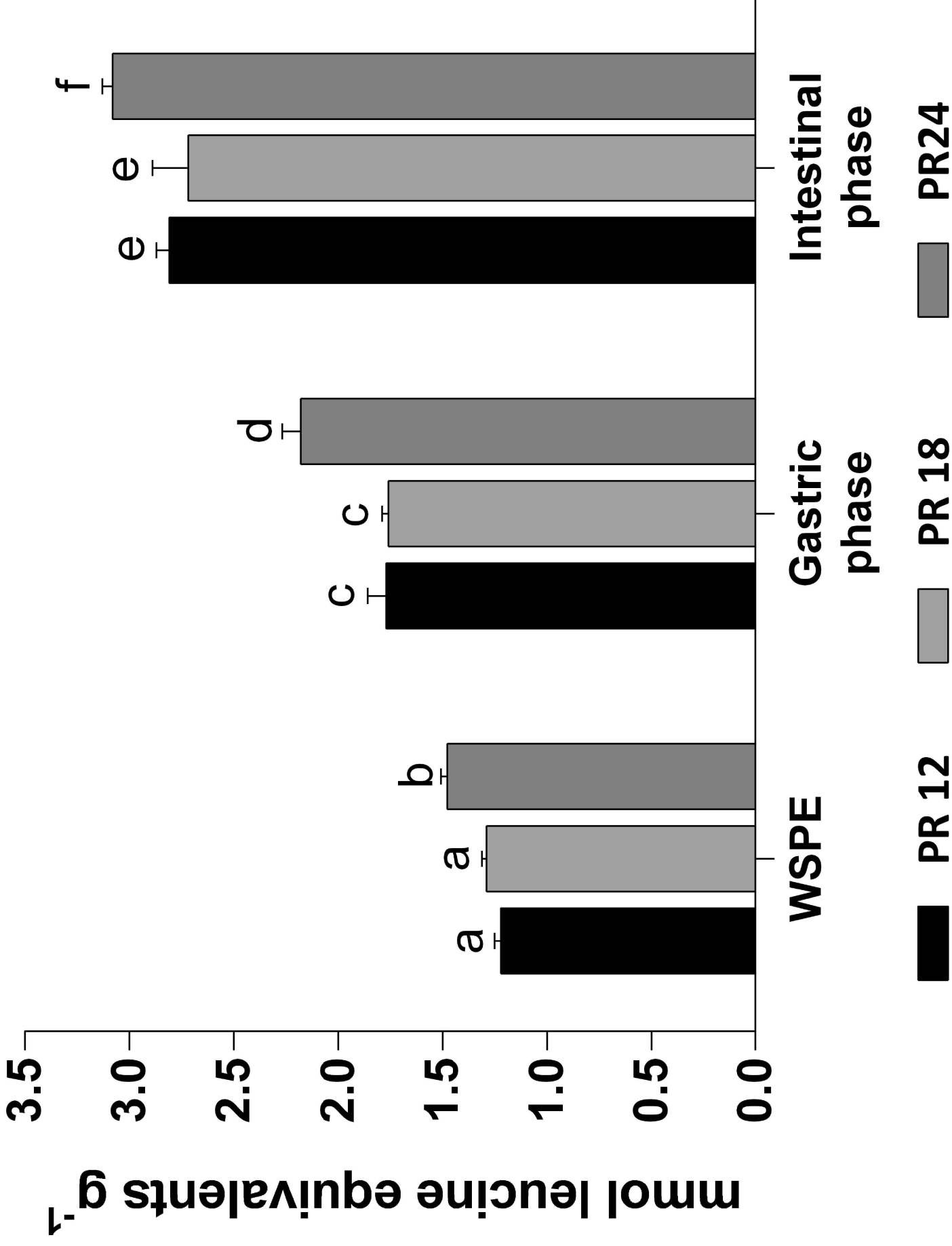
[c]Relative amount was expressed as the area under the peak (AUP)/g of cheese measured from the extracted ion chromatograms (EIC) obtained for each peptide. N.d. means peptide not detected in the sample. Different superscript letters within the same row indicate that the values are significantly different ($P<0.05$).
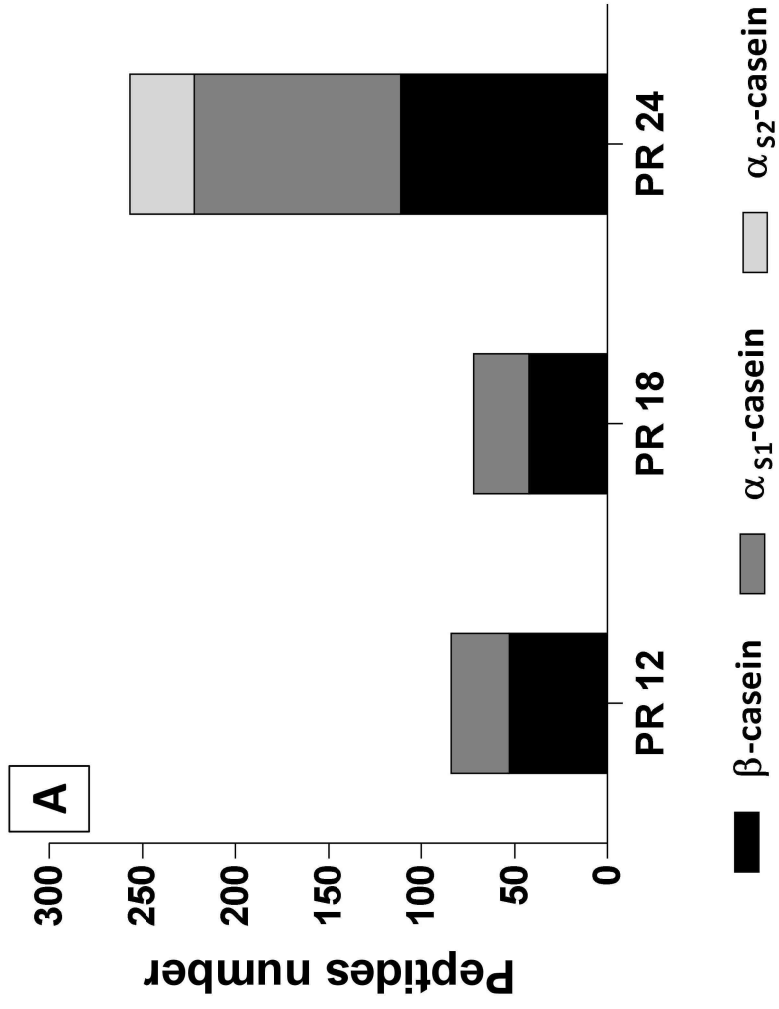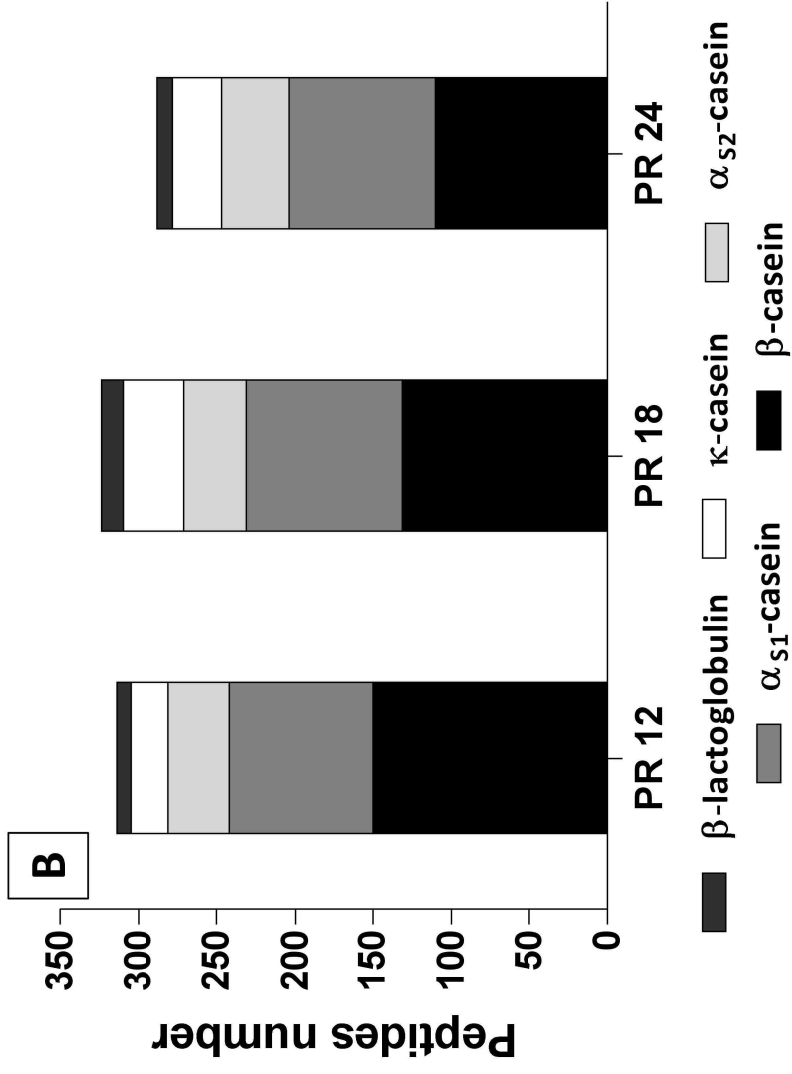
Table 3. Amount of bioactive peptides in water-soluble extract (WSPE) and in digested peptidic fractions from Parmigiano Reggiano samples at 12, 18 and 24 months of ripening

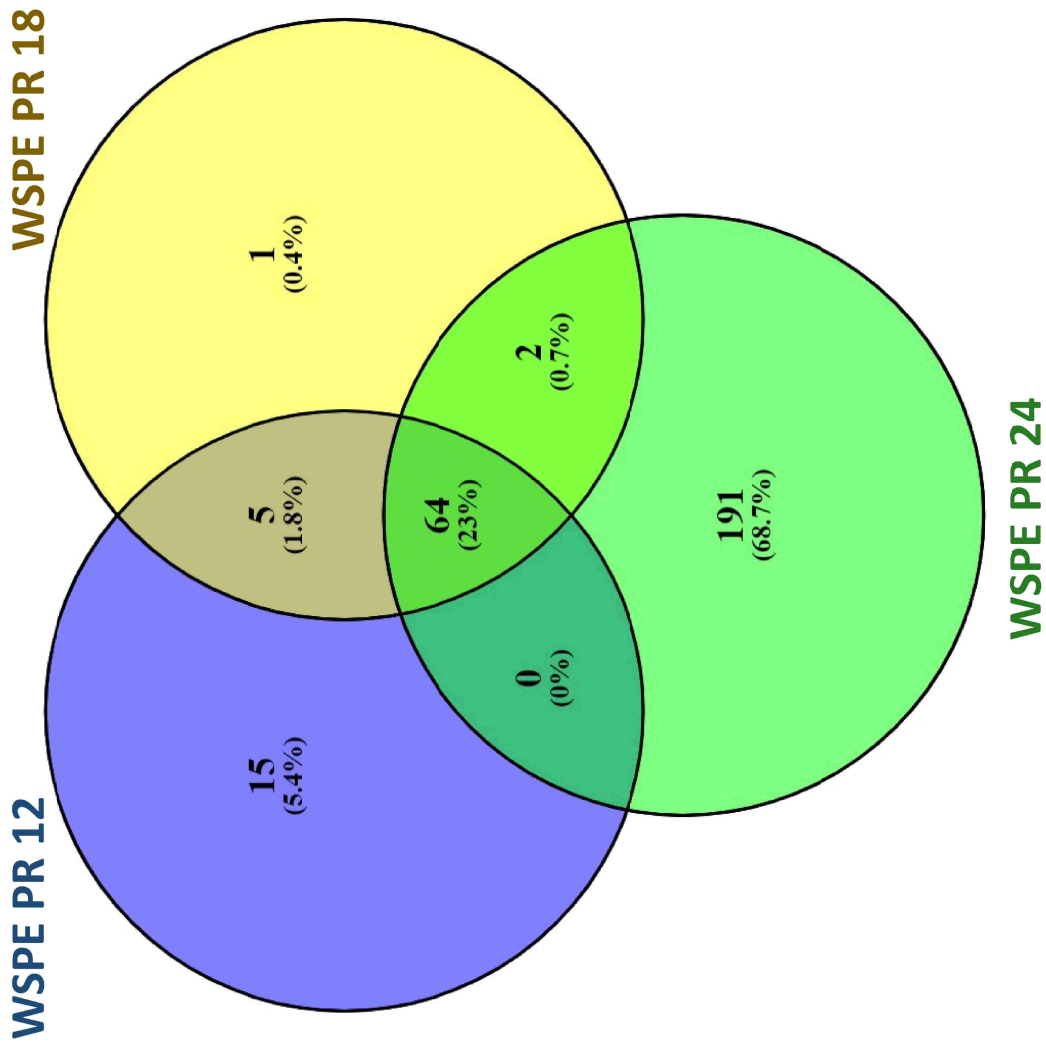| Sequence[a]	WSPE PR12 mg kg^{-1}	WSPE PR18 mg kg^{-1}	WSPE PR24 mg kg^{-1}	Digested PR12 mg kg^{-1}	Digested PR18 mg kg^{-1}	Digested PR24 mg kg^{-1}
VPP	6.87 ± 0.68[a]	11.34 ± 1.01[b]	4.52 ± 0.28[c]	7.73 ± 0.91[a]	12.46 ± 0.97[b]	2.74 ± 0.03[d]
IPP	1.63 ± 0.82[a]	4.24 ± 0.85[b]	0.66 ± 0.05[c]	3.26 ± 0.21[b]	5.64 ± 0.12[d]	0.66 ± 0.23[ac]
YPFPGPI	n.d.	n.d.	n.d.	20.18 ± 3.00[a]	8.91 ± 0.74[b]	6.38 ± 1.39[c]

[a]One code letter was used for amino acid nomenclature.
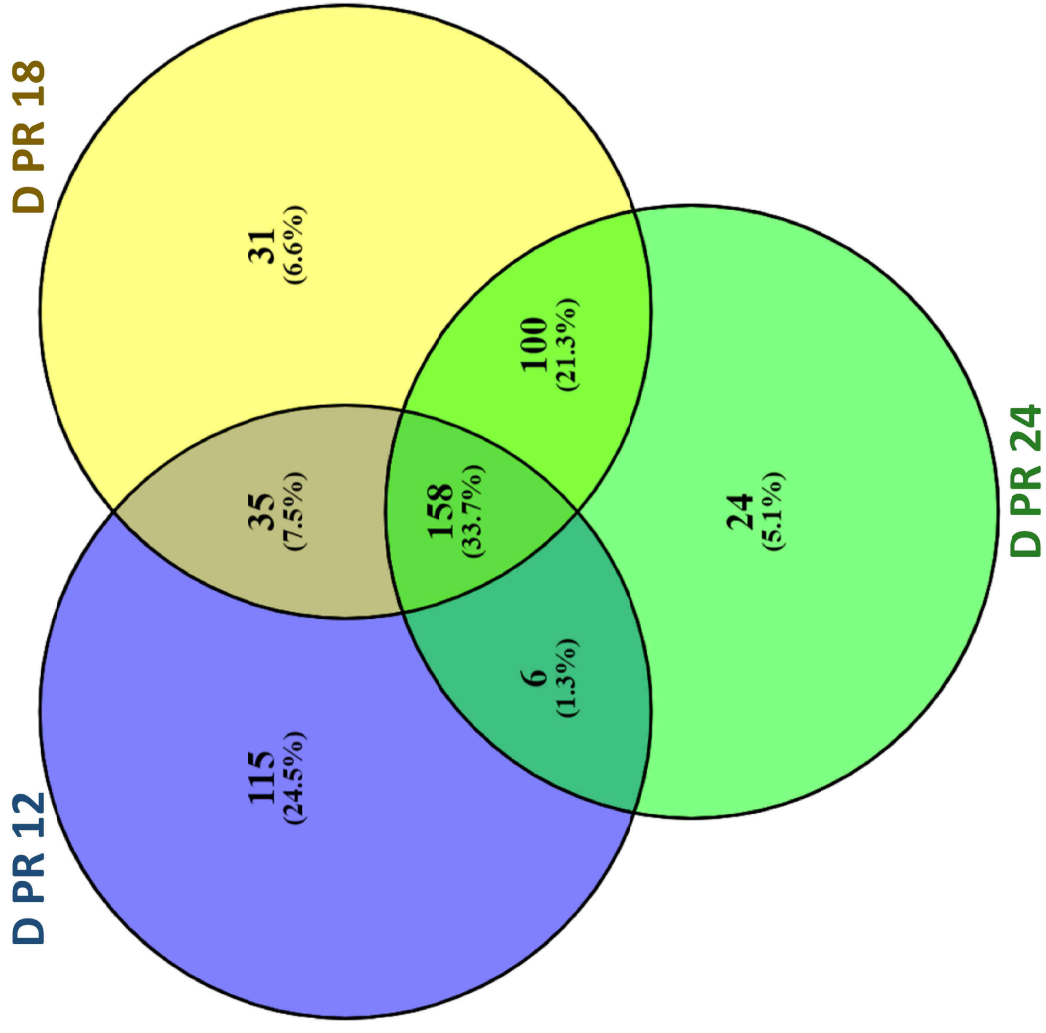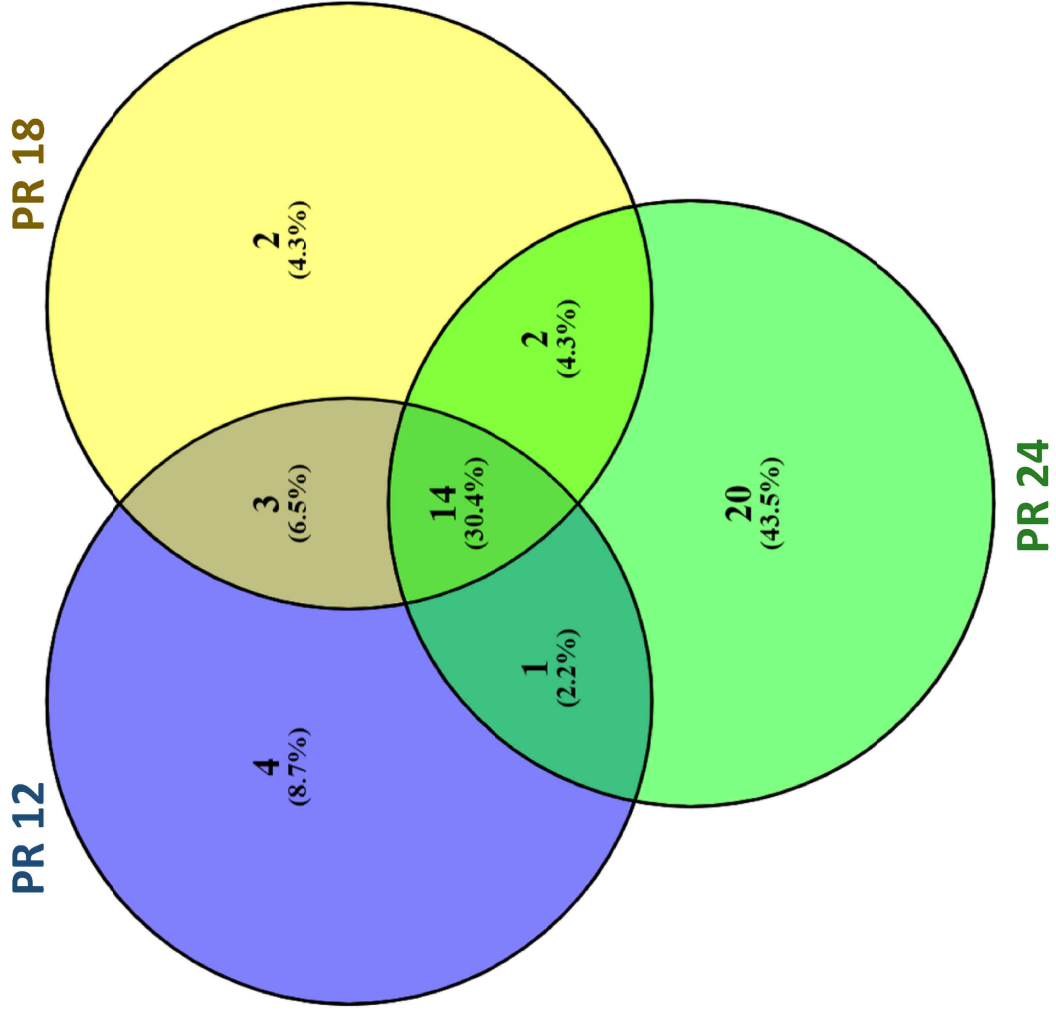N.d. means peptide not detected in the sample

A

WSPE PR 18

WSPE PR 12

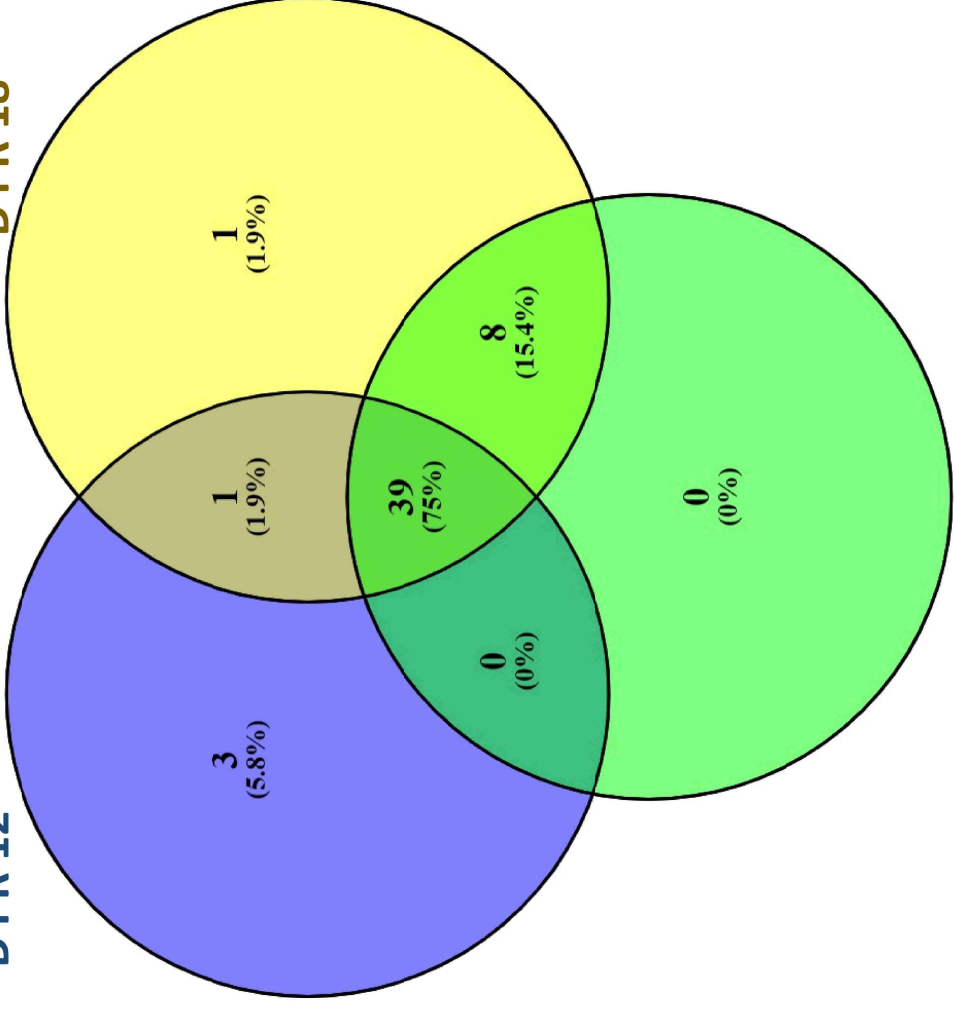WSPE PR 24

15
(5.4%)

5
(1.8%)

1
(0.4%)

0
(0%)

64
(23%)

2
(0.7%)

191
(68.7%)

B

D PR 18

D PR 12

D PR 24

115
(24.5%)

35
(7.5%)

31
(6.6%)

6
(1.3%)

158
(33.7%)

100
(21.3%)

24
(5.1%)

A

WSPE PR 18

WSPE PR 12

0
(0%)

0
(0%)

0
(0%)

11
(42.3%)

1
(3.8%)

0
(0%)

14
(53.8%)

WSPE PR 24

B

D PR 18

D PR 12

1
(1.9%)

1
(1.9%)

3
(5.8%)

39
(75%)

8
(15.4%)

0
(0%)

0
(0%)

D PR 24