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1 **Characterization of the peptide fraction from digested Parmigiano Reggiano cheese and its effect on**  
2 **growth of lactobacilli and bifidobacteria**

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14

15 **Abstract**

16 Parmigiano Reggiano (PR) is a raw-milk, hard cooked, long-ripened cheese of high quality and nutritional  
17 value. Long ripening times allow for extensive proteolysis of milk proteins to yield a number of peptides,  
18 some of which have potential healthy bioactive properties. This study aimed to: i) determine the peptide  
19 profile of PR cheese subjected to simulated gastrointestinal transit; ii) evaluate *in vitro* whether the peptides  
20 could support growth of beneficial microbial groups of the gut microbiota. PR samples were subjected to *in*  
21 *vitro* digestion, simulating oral, gastric, and duodenal transit. Liquid chromatography coupled with tandem  
22 mass spectrometry revealed that digestion caused the disappearance of the serum proteins and most of the  
23 original peptides, while 71 new peptides were found, all ranging from 2 to 24 residues. The digests were  
24 given as sole nitrogen source to pure cultures of *Bifidobacterium* (27 strains) and *Lactobacillus* (30 strains),  
25 and to bioreactor batch cultures of human gut microbiota. Most of bifidobacteria and lactobacilli grew more  
26 abundantly on PR digests than on the control peptone, and exhibited strain- or species- specific peptide  
27 preferences, as evidenced by principal component analysis. Bifidobacteria generally consumed a greater  
28 amount of peptides than lactobacilli, in terms of both the mean peptide consumption and the number of  
29 peptides consumed. For bifidobacteria, peptide preferences were very diverse, but a core of 10 peptides with  
30 4 or 5 residues were consumed by all the strains. Lactobacilli behaved more homogeneously and consumed  
31 nearly only the same 6 peptides, mostly dipeptides. The peptide preferences of the different groups of  
32 bifidobacteria and lactobacilli could not be ascribed to features such as the length of the peptide or the  
33 abundance of residues with peculiar properties (hydrophobicity, polarity, charge) and likely depend on  
34 specific proteases and/or peptide transporters preferentially recognizing specific sequence motifs. The  
35 cultures of human colonic microbiota confirmed that PR digest promoted the growth of commensal  
36 bifidobacteria. This study demonstrated that peptides derived from simulated gastrointestinal digestion of PR  
37 supported the growth of most lactobacilli and bifidobacteria.

38

39

40 **Keywords**

41 Parmigiano Reggiano, Simulated Gastrointestinal Digestion, *Bifidobacterium*, *Lactobacillus*, Intestinal  
42 Microbiota, Bioactive Peptides, Cheese.

43

44

45 **Abbreviations**

46 **PR**, Parmigiano Reggiano; **GIT**, gastrointestinal tract; **SLAB**, starter lactic acid bacteria; **NSLAB**, non-  
47 starter lactic acid bacteria; **PCA**, principal component analysis; **HPLC**, High Performance Liquid

48 **Chromatography**; **UPLC**, Ultra Performance Liquid Chromatography; **ESI**, Electrospray Ionization; **MS**,

49 **mass spectrometry**.

50

51

## 52 **1 Introduction**

53 Parmigiano Reggiano (PR) is a raw-milk, hard cooked, long-ripened cheese of high quality and  
54 nutritional value, produced in a restricted area in northern Italy. PR is produced from bovine milk with rennet  
55 and natural whey starter and a Protected Designation of Origin, in compliance with the European norm  
56 currently in force (European Commission, 2009, 2011). The curd is heated at 55°C to select thermophilic  
57 bacterial strains and it is ripened at least for 12 months, even if much longer ageing times are usually  
58 adopted. In mature PR, the moisture is 28 to 35%. The dry weight is mostly composed of proteins and lipids  
59 fractions, the ratio of fat to protein being around 0.94, depending on milk characteristics. Ripened PR is  
60 lactose- and galactose-free and rich in free organic acids, mostly derived from bacterial fermentations, such  
61 as lactic acid (1.5 g per 100 g of PR), citric (50 mg), acetic (100 mg), propionic (0.5 mg), and butyric (120  
62 mg) acids (Gatti et al., 2014). The overall mineral content of PR is 4.0 to 4.5%, with NaCl at approximately  
63 1.5% (Gatti et al., 2014).

64 Long ripening times allow for cheese extensive proteolysis (Fox and McSweeney, 1998;  
65 McSweeney, 2004; Visser, 1993) due to the residual rennet activity and the enzymes of starter (SLAB) and  
66 non-starter (NSLAB) lactic acid bacteria. During ripening, proteolysis gives rise to the continuous evolution  
67 of oligopeptides and to the release of free amino acids (15 to 25% of protein content), while non-proteolytic  
68 aminoacyl derivatives also accumulate (Sforza et al., 2009, 2012). Casein breakdown contributes to  
69 improved digestibility, reduced allergenicity, and flavor development (Alessandri et al., 2012; Sforza et al.,  
70 2012). The potential biological activity of PR peptides has recently attracted particular interest (Korhonen et  
71 al., 2009; Tidona et al., 2009). *In vitro* studies revealed calcium binding properties (Kim et al., 2004; Pinto et  
72 al., 2012) and antioxidant (Bottesini et al. 2013; Gupta et al., 2009), antihypertensive (Bernabucci et al.,  
73 2014), antimicrobial activities (Benkerroum, 2010; Rizzello et al., 2005)..

74 The nutritional value of PR, coupled with the potential beneficial properties of bioactive peptides  
75 that could impact health, suggested its use as functional food in a dietary therapy for subjects with  
76 inflammatory GIT diseases resulting from alimentary intolerance, post-therapeutic **antibiotic-associated**

77 **dismicrobism**, or post-infective conditions (Olivi et al., 1979; Pancaldi et al., 2008). In particular, a  
78 homemade food based on PR cheese was developed and successfully utilized to feed infants suffering from  
79 different forms of intestinal problems, with a rapid clinical improvement and normalization of **the gut**. The  
80 high digestibility and the high amounts of short chain fatty acids, amino acids, and oligopeptides easily  
81 absorbed in the bowel likely support this beneficial effect. Furthermore, the hypoallergenicity, the absence of  
82 lactose, and the high sodium content that restores losses of salts and reduces the secretions of intestinal cells  
83 have been claimed as responsible of the positive outcome of this dietary therapy (Olivi et al., 1979; Pancaldi  
84 et al., 2008).

85 The human **colon** is colonized by a dense and complex bacterial community exerting important  
86 effects on the health status (Sekirov et al., 2010), wherein the commensal bifidobacteria and lactobacilli are  
87 recognized as beneficial and are commonly utilized as probiotics (Rossi and Amaretti, 2010; Walter, 2008).  
88 **Previous studies demonstrated that the hydrolysates of proteins from different origins, and particularly from**  
89 **milk, can act as growth factors for lactobacilli or bifidobacteria** (Poch and Bezkorovainy, 1991, Ibrahim and  
90 **Bezkorovainy, 1994; Liepke et al., 2002; Oda et al., 2013; Meli et al., 2013, 2014). Moreover, the peptides**  
91 **released with the gastric digestion of an aged cheese were demonstrated to modulate the composition of mice**  
92 **microbiota *in vitro*, improving the abundance of bifidobacteria** (Condezo-Hoyos et al., 2016). On this basis it  
93 has been hypothesized that PR peptides could promote growth and/or activity of these beneficial microbes,  
94 thus modulating the resident microbiota towards a balanced and healthy composition (Sekirov et al., 2010).

95 The health effects of PR bioactive peptides on intestinal bacteria **require** that they resist  
96 gastrointestinal digestion and, at least partially, reach the colon. The present study aimed to characterize the  
97 peptides derived from simulated gastrointestinal digestion of PR cheese, utilizing an *in vitro* digestion model  
98 that recently achieved an international consensus (Minekus et al., 2014). **The resulting peptide mixtures were**  
99 **characterized by liquid chromatography coupled with mass spectrometry. In order to verify whether peptides**  
100 **specifically promoting the growth of beneficial bacteria and potential probiotics could be identified, the**  
101 **misxtures were tested as nitrogen source for bifidobacteria and lactobacilli in pure and fecal cultures.**

102

## 103 **2 Materials and Methods**

### 104 *2.1 Cheese water soluble extract*

105 Samples of PR aged 16, 24, and 36 months (hereinafter referred to as PR16, PR24, and PR36,  
106 respectively) were provided by Consorzio del Formaggio Parmigiano Reggiano (Modena, Italy). Five grams  
107 of finely ground cheese were added with 45 mL of HCl 0.1 M and 1.25 mL of an aqueous solution of  
108 phenylalanyl-phenylalanine (Phe-Phe, 1 mM), that was used as internal standard. Samples were  
109 homogenized for 1 min using an UltraTurrax (IKA T50 basic, Staufen, Germany) at 4000 min<sup>-1</sup>. Insoluble  
110 proteins were removed by acid precipitation followed by centrifugation (45 min at 4°C at 3220 × g),  
111 according to Hernández-Ledesma et al. (2012). The supernatant was filtered through paper filter and  
112 extracted three times with diethyl ether to remove fats. Ether residues were removed with a rotavapor, and  
113 the residual solution was filtered subsequently through 5 µm and 0.45 µm filters.

114

### 115 *2.1 Simulated gastrointestinal digestion of PR samples*

116 All the chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) unless otherwise  
117 stated. The cheese was finely grounded and digested following the procedure, consisting in three main steps:  
118 salivary phase, gastric phase, and intestinal phase (Minekus et al., 2014). A sample of 25 g cheese was added  
119 to 17.5 mL of salivary buffer (15.1 mM KCl, 3.7 mM KH<sub>2</sub>PO<sub>4</sub>, 13.6 mM NaHCO<sub>3</sub>, 0.15 mM MgCl<sub>2</sub>, and  
120 0.06 mM (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>), 2.5 mL of 1500 U/mL amylase, 125 µL of 300 mM CaCl<sub>2</sub>, and 4.875 mL of distilled  
121 water (ratio cheese:digestive fluid of 1:1, w:v). The sample was vortexed and incubated for 2 min at 37°C on  
122 a reciprocating shaker. Then, 37.5 mL of gastric buffer (6.9 mM KCl, 0.9 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>,  
123 47.2 mM NaCl, 0.1 mM MgCl<sub>2</sub>, and 0.5 mM (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>), 8 mL of 25000 U/mL pepsin, 25 µL of 300 mM  
124 CaCl<sub>2</sub>, 1 mL of 1 M HCl and 3.475 mL of water were added (final ratio cheese:digestive fluids 1:3, w:v).  
125 The pH was adjusted to 3 with 1 M HCl. The mixture was vortexed and incubated for 2 h at 37°C on a  
126 reciprocating shaker. Finally, 55 mL of intestinal buffer (6.8 mM KCl, 0.8 mM KH<sub>2</sub>PO<sub>4</sub>, 85 mM NaHCO<sub>3</sub>,

127 38.4 mM NaCl, 0.33 mM MgCl<sub>2</sub>), 25 mL of 800 U/mL pancreatin, 12.5 mL of 75 mg/mL bile solution, 200  
128 μL of 300 mM CaCl<sub>2</sub>, 750 μL of 1 M NaOH and 6.55 mL of distilled water were added (final ratio  
129 cheese:digestive fluids 1:7, w:v). The pH was adjusted to 7 using 1 M NaOH. The sample was vortexed and  
130 incubated for 2 hours at 37°C on a reciprocating shaker. To stop the digestion the sample was heated at 95°C  
131 for 15 min, then cooled. Since the assessment of digestion is strongly affected by the analysis of the digested  
132 fraction, the samples were centrifuged for 45 min at 4°C at 3220 × g to remove insoluble proteins and  
133 undigested components (Minekus et al., 2014). The supernatant was filtered through 0.45 μm membranes.  
134 For chromatographic analysis, 196 μL of sample was supplemented with 4 μL of 1 mM Phe-Phe.

135

## 136 2.2 HPLC-ESI-MS/MS analysis.

137 In order to identify the peptides, the samples were separated by a reverse phase column (Jupiter 5 μm  
138 C18, 90 Å, 2 × 250 mm, Phenomenex, Torrance, CA, USA) in a HPLC (High Performance Liquid  
139 Chromatography) system coupled with electrospray ionization source (ESI) and mass spectrometry (MS)  
140 tandem detector (Alliance 2695 HPLC device with triple quadrupole Quattro micro MS, Waters, Milford,  
141 MA, USA). Elution was performed with the following gradient of eluent A (water with 0.1% formic acid and  
142 0.2% acetonitrile) and eluent B (acetonitrile with 0.1% formic acid): 0-12 min 100% A, 12-77 min from  
143 100% A to 50% A, 77-81 min 50% A, 81-82 min from 50% A to 0% A, 82-90 min 0% A, 90-91 min from  
144 0% A to 100% A, 91-110 min 100% A. The samples were first analyzed in Full Scan mode, to identify the  
145 characteristic ions and the retention time of the unknown compounds, and then in Daughters Scan modality  
146 using a variable collision energy (CE = 10, 20, and 30 eV). HPLC-ESI-MS/MS parameters were: flow 0.2  
147 mL/min; analysis time 110 min; column temperature 35°C; injection volume 40 μL; acquisition time 0-92  
148 min for the water soluble extracts and 7-90 min for the digested samples; ionization type: positive ion mode;  
149 scan range 70-2000 *m/z* (interfering compounds bias was excluded by comparison with a scan range of 100-  
150 2000 *m/z*); capillary voltage 3.2 kV; cone voltage 30 V; source temperature 100°C; desolvation temperature  
151 150°C; cone gas flow 100 L/h; desolvation gas flow 650 L/h.



152 Peptides were manually identified as follows. Molecular ions were recorded from the Full Scan  
153 chromatogram, then they were fragmented in Daughter Scan modality using the triple quadrupole equipment.  
154 The MW of each peptide was launched in FindPept tool (<http://web.expasy.org/findpept> last accessed on  
155 September 2016) against the main milk proteins ( $\alpha$ -casein,  $\beta$ -casein,  $\kappa$ -casein,  $\alpha$ -lactalbumin and  $\beta$ -  
156 lactoglobulin), to obtain a list of peptide sequences with a compatible MW. The theoretical fragmentation  
157 pattern of these sequences was determined  
158 (<http://db.systemsbio.net/proteomicsToolkit/FragIonServlet.html> last accessed on September 2016) and  
159 compared with the MS/MS spectra. The peptide sequences were assigned on the basis of the matching  
160 fragments.

161

### 162 2.3 UPLC/ESI-MS analysis.

163 Absolute quantification of all the peptides identified was not possible due to the unavailability of all  
164 the peptide standards, and a direct comparison of the peptide areas would have been inaccurate due to  
165 differences in ionization efficiency. Once the sequences were assigned, semi-quantitative analysis was  
166 performed using Phe-Phe as internal standard. The ratio between the chromatographic peak of the peptide  
167 and that of Phe-Phe did not yield absolute peptide concentration but allowed the comparison of the same  
168 peptide in different samples. For the semi-quantitative analysis, UPLC (Ultra Performance Liquid  
169 Chromatography) was chosen instead of HPLC, given the higher chromatographic resolution. Samples were  
170 separated by a reverse phase column (Acquity UPLC BEH 300 C18, 1.7  $\mu$ m, 2.1  $\times$  150 mm equipped with a  
171 Acquity UPLC BEH C18 VanGuard Pre-column, 300 $\text{\AA}$ , 1.7  $\mu$ m, 2.1  $\times$  5 mm, Waters) in an UPLC system  
172 coupled with ESI and MS (UPLC Acquity with a single quadrupole detector SQD, Waters). In order to  
173 replicate a chromatographic trace similar to HPLC, but with shorter run time, gradient elution with the same  
174 eluents was programmed as follows: 0-7 min, 100% A; 7-50 min, from 100% A to 50% A; 50-52.6 min, 50%  
175 A; 52.6-53 min, from 50% A to 0% A; 53-58.2 min, 0% A; 58.2-59 min, from 0% A to 100% A; 59-72 min,  
176 100% A. The analysis parameters were: flow 0.2 mL/min; analysis time 72 min; column temperature 35 $^{\circ}$ C;

177 sample temperature 18°C; injection volume 10 µL for water soluble extracts and 2 µL for digested samples;  
178 acquisition time 0-58.2 min for water soluble extracts and 7-58.2 min for digested samples; ionization type:  
179 positive ion mode; capillary voltage 3.2 kV; cone voltage 30 V; source temperature 150°C; desolvation  
180 temperature 350°C; cone gas flow 100 L/h; desolvation gas flow 650 L/h. Samples were analyzed in the Full  
181 Scan mode, with a scan range of 100-2000 *m/z*. The ions of interest were integrated using MassLynx  
182 software (4.0) and semi-quantified using Phe-Phe area in order to compare relative peptide intensities.

183

#### 184 *2.4 Strains and culture conditions*

185 *Bifidobacterium* strains were obtained from the collection of the Dept. of Life Sciences (University  
186 of Modena and Reggio Emilia, Italy), the Scardovi Collection of Bifidobacteria (Buscob, University of  
187 Bologna, Italy), or the American Type Culture Collection (ATCC, USA). *Lactobacillus* strains were obtained  
188 from the collection of Dept. of Food Science (University of Parma, Italy). Bifidobacteria and lactobacilli  
189 were routinely cultured anaerobically at 37°C in Lactobacilli MRS broth (BD Difco, Sparks, NV, USA). For  
190 culturing bifidobacteria, MRS broth was supplemented with 0.5 g/L cysteine hydrochloride.

191

#### 192 *2.5 Pure culture conditions*

193 Utilization of PR digests was investigated in a basal medium (hereinafter referred to as BM) where  
194 PR16, PR24, PR36 digests or Bacto peptone (BD Difco) were utilized as nitrogen source, the latter as  
195 positive control. The medium contained lactose, 10 g/L; PR hydrolysates or Bacto peptone (BD Difco), 5  
196 g/L; ammonium citrate, 2 g/L; yeast nitrogen base w/o amino acids and ammonium sulfate (BD Difco), 1.7  
197 g/L; polysorbate 80, 1 g/L; sodium acetate trihydrate, 5 g/L; K<sub>2</sub>HPO<sub>4</sub>, 2 g/l; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.1 g/l; MnSO<sub>4</sub> ·  
198 H<sub>2</sub>O, 0.05 g/L; cysteine hydrochloride, 0.5 g/l; pH was corrected to 6.5. Bacteria inoculated (5% v/v) in BM  
199 based media were incubated 24 h at 37°C, and passaged at least three times before evaluation of growth and  
200 peptide consumption.

201

Growth of bifidobacteria was determined in triplicate by measuring the turbidity at 600 nm (OD<sub>600</sub>).

202 at the beginning and after 24 h of incubation. Growth of lactobacilli was evaluated in triplicate using a  
203 BacTrac 4300 Microbiological Analyzer system (Sylab, Neupurkersdorf, Austria). The BM culture was  
204 diluted 1:100 in Ringer's solution (Oxoid, Basingstoke, UK), then 0.1 ml were used to inoculate tubes  
205 containing 6 mL of BM media. The impedance measurement was performed at 37°C. Bacteria growth was  
206 measured as E%, where E-value is the impedance change revealed by the system at the electrode surface.  
207 Variation in electrical conductivity, monitored during time, is proportional to the change in the number of  
208 microorganisms (Bancalari et al., 2016). Maximum value of E% was recorded as index of maximum  
209 microbial growth.

210

## 211 2.6 Cultures of intestinal microbiota

212 Batch cultures of fecal microbiota were performed in bioreactors containing 200 mL of FM medium,  
213 developed from the media described by Walker et al. (2005) and Duncan et al. (2002), with some  
214 modifications: beech wood xylan, 0.6 g/L; citrus pectin, 0.6 g/L; maize amylopectin, 0.6 g/L; larch wood  
215 arabinogalactan, 0.6 g/L; potato starch, 3 g/L; fructans (Synergy1, Beneo, Mannheim, Germany), 3 g/L;  
216 peptone, 5 g/L; ammonium citrate, 2 g/L;  $\text{KH}_2\text{PO}_4$ , 2 g/L; NaCl, 4.5 g/L;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g/L;  $\text{CaCl}_2 \cdot$   
217  $2\text{H}_2\text{O}$  0.045 g/L;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005 g/L; hemin, 0.01 g/L; bile salts (Oxgall, BD Difco) 0.05 g/L,  
218 resazurin, 0.6 mg/L; reducing solution, 40 ml/L; mineral solution, 2 ml/L; vitamin solution, 1.4 ml/L.  
219 Reducing solution contained: cysteine hydrochloride, 12.5 g/L;  $\text{NaHCO}_3$ , 80 g/L. Mineral solution  
220 contained: EDTA, 500 mg/L;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 200 mg/L;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 10 mg/L;  $\text{MnCl}_2 \cdot 7\text{H}_2\text{O}$ , 3 mg/L;  
221  $\text{H}_3\text{BO}_3$ , 30 mg/L;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 20 mg/L;  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , 1 mg/L;  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 2 mg/L;  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 3  
222 mg/L. Vitamin solution contained: menadione, 1 g/L; biotin 2 g/L; calcium pantothenate 2 g/L; nicotinamide,  
223 10 g/L; vitamin B12, 0.5 g/L; folic acid 0.5 g/L; thiamine, 4 g/L; PABA, 5 g/L. Reducing, mineral and  
224 vitamin solutions were filter sterilized and added to the autoclaved medium. PR24 digest or Bacto peptone  
225 were utilized as nitrogen source at the concentration of 5 g/L in parallel batches, carried out in duplicate. The  
226 inoculum was prepared pooling equal amounts of fecal microbiota from three healthy adult subjects. The

227 feces were homogenized (5% w/v) with the medium in the anaerobic cabinet (Anaerobic System, Forma  
228 Scientific, Marietta, OH) under a 85% N<sub>2</sub>, 10% CO<sub>2</sub>, 5% H<sub>2</sub> atmosphere. 10 mL of the suspension were  
229 inoculated into bioreactors (Sixfors V3.01, Infors, Bottmingen, Switzerland) each containing 190 mL of  
230 fresh sterile medium, and the processes proceeded for 48 h (37°C, CO<sub>2</sub> atmosphere, pH 6.2, stirring 60 rpm).  
231 5 mL samples were collected at 0, 6, 12, 24 h and stored at -20°C until analyzed.

232 The abundance of total eubacteria, bifidobacteria and lactobacilli was determined by qPCR  
233 with the following primer pairs: F-Eub/R-Eub (TCCTACGGGAGGCAGCAGT/  
234 GGACTACCAGGGTATCTAATCCTGTT) (Nadkarni et al., 2002), BiTOT-F/BiTOT-R  
235 (TCGCGTCYGGTGTGAAAG/CCACATCCAGCRTCCAC) (Rinttilä et al., 2004), and Lac-F/Lac-R  
236 (GCAGCAGTAGGGAATCTTCCA/GCATTYCACCGCTACACATG) (Castillo et al., 2006). DNA  
237 extraction and amplification reactions were performed according to Simone et al. (2014). The following  
238 thermocycle was utilized for eubacteria and lactobacilli: 98°C for 2 min; 40 cycles of 98°C for 5 s, 64.5°C  
239 for 5 s, and 95°C for 1 min; 65°C for 1 min. The same program was utilized for bifidobacteria, with  
240 annealing temperature of 62.5°C.

241

## 242 *2.7 Principal component analysis of peptide consumption*

243 Principal component analysis (PCA) (Massard et al., 1997; Wold et al., 1987) was carried out to  
244 compare the peptide consumption by bifidobacteria and lactobacilli. Due to matrix effects resulting from  
245 components of the medium, consumption could be evaluated only for 63 out of the 72 peptides occurring in  
246 the extracts. The experimental data were analyzed using PLS Toolbox software ver. 8.1.1 (Eigenvector  
247 Research Inc., Wenatchee, WA, USA) for the calculation of PCA models. A PCA model was calculated on a  
248 dataset with size {45, 63} including all the 45 samples (i.e. 15 strains × 3 ripening ages of PR) obtained by  
249 digestions carried out with bifidobacteria and lactobacilli, each one characterized by the consumption values  
250 of 63 peptides. Two further PCA models were calculated considering bifidobacteria and lactobacilli  
251 separately, i.e. on two separate datasets with size equal to {24, 63} and to {21, 63}, respectively. Before

252 calculating the PCA models, the variables were pre-processed using mean centering. The number of  
253 significant PCs, i.e., the number of PCs bearing useful information, was selected based on the scree plot  
254 reporting the percentage of variance explained by each PC vs. the PC number.

255

### 256 **3 Results**

#### 257 *3.1 Effects of simulated digestion on the peptide profile of PR*

258 The peptide profile of PR16, PR24, and PR36 was characterized by HPLC/ESI-MS/MS before and  
259 after the *in vitro* simulated gastrointestinal digestion (Figure 1A, 1B). Intact caseins are not taken into  
260 account, since they were removed from both the water soluble extracts and from the digested samples.  
261 Before the simulated digestion, a common peptide profile was detected in the water extracts of PR16, PR24,  
262 and PR36 samples (Fig. 1A). 63 main peptides were identified, including some N-lactosyl amino acids, with  
263 a length up to 103 residues and a molecular weight spread over a wide range (Supporting Information: Table  
264 S1). 31 peptides had a MW < 500 Da, 2 had a MW of 500-1000 Da, 12 fell in the range 1000-2000 Da.  
265 Longer peptides were also identified, 12 with MW of 2-5 kDa and at 6 with MW of 5-20 kDa. Moreover, the  
266 two isoforms of  $\beta$ -lactoglobulin (A and B) were clearly detectable at 40.14 min.

267 After *in vitro* digestion, the mixtures of PR16, PR24, and PR36 still shared similar profile. The  
268 digests lacked intact proteins, including  $\beta$ -lactoglobulins, and 61 out of the 63 peptides identified in water  
269 soluble extracts were no longer detectable, being degraded into shorter ones (Fig. 1B). The sole exception  
270 were FVAPFPEVFGK and the phosphopeptide LEELNVPGEIVESLSSS. The former was present in the  
271 water soluble extract, especially of the PR36, and remained in similar amounts after digestion, whereas the  
272 latter occurred in the digests in minor amount. N-lactosyl amino acids also resisted digestion, but their  
273 chromatographic signal was negligible, compared with those of peptides.

274 As a whole, 72 main peptides were identified in the PR digests (Supporting Information: Table S2)  
275 and, with the exception of P3, P13, P71, and P72, were assigned to specific sequences. Compared with the  
276 undigested water extracts, molecular weight distribution of the peptides was shifted toward lower MW: 12

277 peptides had MW < 500 Da, 41 had a MW of 500-1000 Da, constituting the majority of peptides of the  
278 digests, and 14 peptides had a MW of 1000-2000 Da. Only 3 peptides presented a MW > 2 kDa, none of  
279 them exceeding 2700 Da. (Fig. 1B).

### 281 3.2 Growth of bifidobacteria and lactobacilli on PR digests as nitrogen source

282 Twenty seven *Bifidobacterium* strains belonging to the species *B. adolescentis*, *B. animalis* subsp.  
283 *animalis*, *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*,  
284 and *B. pseudocatenulatum*, and 30 *Lactobacillus* strains belonging to the species *L. casei*, *L. paracasei* and  
285 *L. rhamnosus* were investigated for the ability to grow utilizing PR hydrolysates as nitrogen source. All the  
286 strains grew both in the control medium and the PR-based media (Fig. 2), exhibiting a species specific  
287 growth yield, with few exceptions. Within the phylogenetically related group of *B. adolescentis* and *B.*  
288 *pseudocatenulatum*, the strains belonging to the former species gave similar yields in the diverse BM media,  
289 while the strains belonging to the latter grew more abundantly with PR digests. All the strains of *B. breve*  
290 and most of *B. longum* grew more abundantly in the media containing the PR digests than in the control. On  
291 the contrary, most *B. bifidum* and *B. animalis* grew more abundantly using the control peptone as nitrogen  
292 source than PR digests. *L. casei* grew more abundantly on PR digests, while *L. paracasei* 2306 and the *L.*  
293 *rhamnosus* strains 2323, 2303, 2302, 2409 grew better on the control peptone. A trend common to all the  
294 tested species and strains, with respect to the growth and the age of PR, could not be found. However, most  
295 of the bifidobacteria that preferred PR digests over the control peptone grew more abundantly on PR36 or  
296 PR24 than on PR16. On the other hand, most of the lactobacilli preferring PR digests over the peptone, grew  
297 better on PR16 or PR24 than on PR36.

### 299 3.3 Consumption of PR peptides by bifidobacteria and lactobacilli

300 Peptide utilization was analyzed by UPLC/ESI-MS for 8 *Bifidobacterium* and 7 *Lactobacillus*  
301 strains, representative of different species/subspecies and selected among those with best growth on PR

302 digests, (Fig. 3). Peptide profiles of cultures grown for 24 h on BM medium supplemented with PR16, PR24,  
303 or PR36 digests were compared with the profiles of non-inoculated media incubated under the same  
304 conditions. The percentage of consumption of each peptide was determined (Fig. 3) and the consumption  
305 profiles were compared by PCA (Fig. 4).

306 Bifidobacteria consumed a greater number of peptides and at a greater extent than lactobacilli (Fig.  
307 3). Thus, bifidobacteria and lactobacilli clustered separately in the score plot of the first 2 PCs, which  
308 explains 66% of total data variance (Fig 4a). Lactobacilli are closely grouped, while bifidobacteria are much  
309 more dispersed, indicating that lactobacilli behaved more homogeneously than bifidobacteria with respect to  
310 peptide utilization. Based on the corresponding loading plot (Fig. 4b), consumption of peptides P4, P9, P12,  
311 P18, P20, P48, P49, P68, and P72 characterized the behavior of lactobacilli, whereas peptide consumption  
312 was more diversified among bifidobacteria.

313 Bifidobacteria presented different patterns of utilization (Fig. 3). All the strains nearly depleted P1,  
314 P27, P28, P30, and P45 while they did not consume, or scarcely consumed, P39, P53, P69, and P70.  
315 However, preferential utilization or non-utilization of most peptides was associated to one or a group of  
316 strains, as revealed by the PCA model calculated on bifidobacteria (Fig. 5a, 5b). The score plot of the first 2  
317 PCs (accounting for 64% of total data variance) shows that bifidobacteria are clearly grouped (Fig. 5a) on the  
318 basis of their peptide preferences observable in the corresponding loading plot (Fig. 5b). *B. adolescentis*  
319 RBB4 (B1), located distant from other bifidobacteria along PC1 (Fig. 5a), was the most efficient in utilizing  
320 PR peptides. It consumed 45-47 peptides for more than 75% and 21-23 for more than 95% (Fig. 3).  
321 Moreover, it was the sole strain able to consume specific peptides, such as P14, P25, P29, P47, P48, P66,  
322 P67, P68, including the diphosphopeptide P44 (Fig. 3).

323 *B. animalis* subsp. *animalis* ATCC 27536 (B3) and *B. animalis* subsp. *lactis* WC 0413 (B4) were the  
324 less efficient and grouped together in the PC1-PC2 score plot (Fig. 5A). They consumed only 8-10 peptides  
325 for more than 75%, none of them for more than 95% (Fig. 3). The strains of *B. animalis* strains consumed  
326 P33, while utilized less efficiently several peptides generally consumed by the other bifidobacteria (e.g. P12,

327 P15, P16, P19, P24, and P38) (Fig. 3).

328 *B. bifidum* B2091 (B5) and *B. breve* WC 0423 (B6) behaved similarly and differed from the other  
329 bifidobacteria along PC3 (PC3 and PC4 account for further 21% of total data variance), mostly because of  
330 greater consumption of P12, P18, P26, P33, P35, P36, P38, P57, and P71 (Fig. 3, Supporting Information:  
331 Fig. S1).

332 *B. longum* strains (B7 and B8) shared a similar behavior towards many peptides, but *B. longum*  
333 subsp. *infantis* WC 0438 (B7) was able to consume also P2, P7, P23, P52, P57, P59, P62, P63, P64, and P71.  
334 This explains the strains not grouping together, with a clear separation along PC1, PC2, (Fig. 5a) and PC4  
335 (Supporting Information: Fig. S1). Peptide consumption by *B. longum* subsp. *longum* WC 0438 (B8) and *B.*  
336 *pseudocatenulatum* WC 0403 (B2) was very similar, being largely overlapped in the first three principal  
337 components and slightly differentiating only along PC4 (Supporting Information: Fig. S1).

338 Lactobacilli were less performing in PR peptide consumption compared to bifidobacteria (Fig. 3).  
339 They all consumed P1, P4, P9, P12, P18, and P20, but generally did not use, or used at lower extent, the vast  
340 majority of the other peptides. The first 2 PCs of the PCA model calculated on lactobacilli (accounting for  
341 61% of total data variance) showed that the pattern of peptide consumption was homogenous for most of the  
342 lactobacilli, with minor differences. The major differences were observed for *L. casei* 2414 (L6) and *L.*  
343 *paracasei* 2306 (L7) (Fig. 5c) that clustered separately along PC1 and were characterized by high  
344 consumption of P15, P46, P49, P57, and P64 and by poor utilization of P13, P27, P68, and P72 (Fig. 5d).  
345 P68 and P72 were consumed for more than 60% by all the lactobacilli, with the exception of *L. casei* 2414  
346 (L6) and *L. paracasei* 2306 (L7).

347 All the other lactobacilli clustered together at high values of PC1 and presented a similar pattern of  
348 peptide utilization. *L. rhamnosus* 2232 (L1), *L. rhamnosus* 2325 (L2), *L. rhamnosus* 2362 (L3), *L. casei* 2240  
349 (L4), and *L. casei* 2404 (L5) are distributed along PC2. *L. rhamnosus* 2232 (L1) lies at positive values of  
350 PC2, while *L. rhamnosus* 2325 (L2) and *L. casei* 2404 (L4) are found at negative PC2 values (Fig. 5c). In  
351 fact, despite their pattern of utilization was very similar, *L. rhamnosus* 2232 (L1) was the most efficient in



352 peptides utilization, with 10 peptides consumed for more than 75%, and differed especially for a greater  
353 consumption of P16 and few other peptides (Fig. 3 and Fig. 5d). On the contrary, *L. rhamnosus* 2325 (L2)  
354 and *L. casei* 2404 (L5) were the least performing in PR peptides consumption, with only the most 6 peptides  
355 consumed by all the strains utilized for more than 75% (Fig. 3).

356 The score plots of both bifidobacteria and lactobacilli did not exhibit any grouping and/or  
357 distribution along the principal components, based on the extent of the growth of the bacteria. Similarly, also  
358 the loading plots of both bifidobacteria and lactobacilli did not exhibit any trend along the principal  
359 components, based on the main characteristic of the peptides, such as the length, the abundance of  
360 hydrophobic, polar, and positively/negatively charged amino acids (data not shown), or the presence of  
361 sulfur-containing residues.

362

### 363 3.4 Microbiota cultures

364 Cultures of intestinal microbiota were performed in a medium containing 5 g/L of PR24 digest as  
365 only nitrogen source or peptone as control (Fig. 6). In both the cultures, total bacteria similarly increased by  
366 more than 2 magnitudes in the first 6 h of incubation, then remained stationary at a level of approx. 10 Log<sub>10</sub>  
367 cells/mL (Fig. 6A). Bifidobacteria were 6.4 Log<sub>10</sub> cells/mL at the beginning of batch process, accounting for  
368 4-5% of total bacteria. They grew abundantly in the first 6 h, up to 8.7 and 8.4 Log<sub>10</sub> cells/mL in PR24 and  
369 control cultures, respectively. In control cultures bifidobacteria remained stable, representing approx. the 5%  
370 of eubacteria. In PR24 cultures, they increased up to 8% after 12 h and 14% after 24 h, even though in this  
371 latter time-point the higher abundance arose also from the slight decrease in total bacteria. Unlike  
372 bifidobacteria, lactobacilli never appeared above the limit of detection of 4 Log<sub>10</sub> cells/mL. Most of the  
373 peptides of PR24 digest were consumed in the first 6 h of incubation and completely disappeared in the  
374 following incubation hours (Fig. 6B).

375

## 376 4 Discussion

377 The present study aimed to characterize the peptides that are expected to escape digestion of PR and  
378 to investigate whether they could support the growth of beneficial bacteria in the colon. The water soluble  
379 extracts of PR contained a few intact proteins (e.g. the two isoforms of  $\beta$ -lactoglobulin) and a variety of  
380 peptides of 2 to 103 residues originated during ripening through the proteolytic cleavage of cheese proteins  
381 (mainly  $\alpha$  and  $\beta$ -casein) by rennet, SLAB, and NSLAB (McSweeney, 2004). The water extracts of PR16,  
382 PR24, and PR36 shared similar profile, consistently with previous evidence that the most relevant  
383 proteolytic transformations occur in the first 12 months, before the PR can be sold on the market (Sforza et  
384 al. 2008).

385 Few previous studies performed *in vitro* digestion of PR, but mainly focused on quantification of  
386 specific peptides (Bordoni et al., 2011; De Noni and Cattaneo, 2010; Basiricò et al., 2015), without providing  
387 a thorough characterization of the whole profile. The main peptide composition of PR after simulated  
388 digestion is reported herein for the first time. The PR digests lacked serum proteins and any peptide longer  
389 than 24 residues. The new peptides derived from the hydrolysis of the high molecular weight peptides  
390 already present in cheese and from the breakdown of intact  $\alpha$ - and  $\beta$ -casein and whey proteins. Any  
391 predictable and specific cleavage sites could be found in peptide sequences, consistently with the fact that the  
392 proteolytic activity of pancreatin is due to trypsin, chymotrypsin, but also elastase, carboxypeptidase and  
393 other minor proteases, and suggesting that many peptides are generated by the action of an exopeptidase or  
394 from aspecific cleavages.

395 Some peptides identified in the digests bear sequences of biological interest. Nonetheless, it is not  
396 clear whether they could be effectively active, since the sequences were found within longer peptides for  
397 which the biological activity has not been investigated so far. In particular, the sequences of the opioids  $\beta$ -  
398 casomorphin 5 (YFPFG) and  $\beta$ -casomorphin 7 (YFPFGPI) and the ACE inhibitor peptide  $\beta$ -casomorphin 9  
399 (YFPFGPIP) were found within longer peptides originating from  $\beta$ -casein, such as P50, P56, and P58  
400 (Nguyen et al, 2015). The sequences of the ACE inhibitor tripeptides VPP and IPP were similarly found  
401 within longer peptides of the digests, such as P63, P69, and P70. The ACE inhibitor sequence  $\alpha_{s1}$ -cn f(24-

402 32) (FVAPFPEVF) was found within peptide P59. Other bioactive peptides which could occur in PR were  
403 no longer detectable after digestion, thus some doubts arise about their real availability *in vivo*.

404 Potential health promoting effects of PR peptides are supported by observation that the digests of PR  
405 sustained abundant growth of pure cultures of bifidobacteria and lactobacilli, natural beneficial colonizers of  
406 the human colon and potential probiotics (Rossi and Amaretti, 2010; Walter, 2008). Bifidobacteria were  
407 more efficient in consuming PR peptides than lactobacilli, with a greater number of peptides consumed at a  
408 greater extent. Peptide preferences of bifidobacteria were specific for each strain, as evidenced by PCA, with  
409 a core of peptides with 4 or 5 residues consumed by all strains (P1, P27, P28, P30, and P45). On the other  
410 hand, lactobacilli behaved very homogeneously and seemed to prefer the same peptides, mostly di- and  
411 tripeptides, such as P4, P9, P12, P18, and P20. The differences among lactobacilli were minor and restricted  
412 to a limited number of peptides, such as P64, P68, and P72. The lower peptides consumption by lactobacilli  
413 is consistent with the ability of NSLAB to use also free amino acids and other nitrogen compounds derived  
414 from nucleic acids and casein degradation (Kieronczyk, 2001; Sgarbi et al., 2014). Despite PCA enabled  
415 clustering bacteria on the basis of peptide utilization, no grouping and/or distribution of the peptides could be  
416 established based on their main characteristics. In fact, the peptide preferences of the different groups of  
417 bifidobacteria and lactobacilli could not be ascribed to features such as the length of the peptide, the presence  
418 of sulfur-containing amino acids, or the abundance of residues with peculiar properties (hydrophobicity,  
419 polarity, charge) and likely depend on specific proteases and/or peptide transporters preferentially  
420 recognizing specific sequence motifs in the peptides.

421 The bifidogenic effect of PR digests in pure and microbiota cultures is coherent with previous  
422 studies which described the stimulation of bifidobacteria by peptic and/or tryptic digests of certain milk  
423 proteins (Poch and Bezkorovainy, 1991; Oda et al. 2013; Condezo-Hoyos et al. 2016). In microbiota  
424 cultures, the digest of PR24 promoted the growth of bifidobacteria, the counts of which increased in the first  
425 hours of fermentation and achieved a higher relative abundance towards the end of the fermentation,  
426 compared with control cultures. However, cultivation of intestinal microbiota in PR-based medium resulted

427 in rapid and complete consumption of peptides in the first hours of incubation, confirming the intense  
428 breakdown and assimilation of peptides by the dense proteolytic population of the colon (Richardson et al.,  
429 2013). Unlike bifidobacteria, the population of lactobacilli never increased in microbiota cultures over the  
430 limit of detection of qPCR quantification (4 Log<sub>10</sub> cells/mL), likely because resident lactobacilli represent a  
431 minority population within the human gut microbiota (Quartieri et al., 2016; Rossi et al., 2016).

432

## 433 **5 Conclusions**

434 The present study describes several new, previously not reported, peptides originating from cheese  
435 digestion, thus opening the question whether they are bioactive. Determining the bioactivity of the peptides  
436 of PR digests is attractive and deserves further studies. Despite the limitation of the experimental procedure,  
437 that did not take into account the absorption in the small intestine, it was demonstrated that PR digests  
438 promoted the growth of bifidobacteria both in pure and in microbiota cultures. This observation strengthens  
439 the potential impact on the microbiota as a potential mechanism of health promotion of PR dietary  
440 supplementation, even though the highly proteolytic environment and the strong and unpredictable  
441 competition among several other bacteria make impossible to predict what can happen *in vivo*. The  
442 interaction between specific peptides and colonic bacteria is still largely unexplored and, based on the results  
443 herein presented, opens intriguing perspective in the modulation of the gut microbiota towards a healthy  
444 composition and deserves deeper investigation.

445

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448

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578 **Figure legends**

579 **Figure 1.** Overlaid Full Scan chromatograms obtained for the waters soluble extracts (**Panel A**) and the  
580 digests (**Panel B**) of PR16 (black), PR 24 (green), and PR 36 (orange). Chromatograms were obtained by  
581 UPLC/ESI-MS and they are showed as Total Ion Current (TIC); run time: 72 min.

582 **Figure 2.** Growth of 27 strains of *Bifidobacterium* (**Panel A**) and 30 of *Lactobacillus* (**Panel B**) in BM  
583 medium containing 5 g/L control peptone (grey) or PR the digests from PR16 (yellow), PR24 (orange), and  
584 PR36 (red). Growth of bifidobacteria and lactobacilli is reported as the increase of turbidity (OD<sub>600</sub>) and  
585 impedance (E%), respectively. Values are means, n = 3, SD < 0.1. The box and whiskers plots (**Panel C**)  
586 report, for each species or group of species/subspecies, the increase in the different media. Boxes indicate the  
587 median and 25<sup>th</sup> and 75<sup>th</sup> percentiles; whiskers indicate the minimum and the maximum.

588 **Figure 3.** Peptide consumption by *Bifidobacterium* and *Lactobacillus* strains cultured for 24 h in BM  
589 medium containing 5 g/L of PR16, PR24, and PR36 digests. Percentage consumption, relative to non-  
590 inoculated media similarly incubated for 24 h at 37°C, are reported as colors ranging from the lowest  
591 (deepest green) to the highest (deepest red) consumption. Values are means, n = 3, SD always < 7.

592 **Figure 4.** PCA model of peptide consumption by *Bifidobacterium* and *Lactobacillus* strains: PC1-PC2 score  
593 plot (**a**) and loading plot (**b**).

594 **Figure 5.** PCA model calculated on *Bifidobacterium* strains: PC1-PC2 score plot (**a**) and loading plot (**b**);  
595 PCA model calculated on *Lactobacillus* strains: PC1-PC2 score plot (**c**) and loading plot (**d**).

596 **Figure 6.** Cultures of human intestinal microbiota with PR24 digests. **Panel A:** time-course of total  
597 eubacteria and bifidobacteria in FM medium supplemented with 5 g/L of PR24 digests (orange) or control  
598 peptone (grey). Bacteria were quantified by qPCR. Symbols: total eubacteria, dotted line; bifidobacteria,  
599 dashed line; relative amount of bifidobacteria in the microbiota, solid line. Values are means ± SD, n = 3.

600 **Panel B:** Overlaid chromatograms of PR24 cultures of intestinal microbiota at 0 (black), 6 (green), and 12 h  
601 (yellow) of incubation. Chromatogram was obtained by UPLC/ESI-MS and they are showed as Total Ion  
602 Current (TIC); run time: 72 min.

1 **Characterization of the peptide fraction from digested Parmigiano Reggiano cheese and its effect on**  
2 **growth of lactobacilli and bifidobacteria**

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14

15 **Abstract**

16 Parmigiano Reggiano (PR) is a raw-milk, hard cooked, long-ripened cheese of high quality and nutritional  
17 value. Long ripening times allow for extensive proteolysis of milk proteins to yield a number of peptides,  
18 some of which have potential healthy bioactive properties. This study aimed to: i) determine the peptide  
19 profile of PR cheese subjected to simulated gastrointestinal transit; ii) evaluate *in vitro* whether the peptides  
20 could support growth of beneficial microbial groups of the gut microbiota. PR samples were subjected to *in*  
21 *vitro* digestion, simulating oral, gastric, and duodenal transit. Liquid chromatography coupled with tandem  
22 mass spectrometry revealed that digestion caused the disappearance of the serum proteins and most of the  
23 original peptides, while 71 new peptides were found, all ranging from 2 to 24 residues. The digests were  
24 given as sole nitrogen source to pure cultures of *Bifidobacterium* (27 strains) and *Lactobacillus* (30 strains),  
25 and to bioreactor batch cultures of human gut microbiota. Most of bifidobacteria and lactobacilli grew more  
26 abundantly on PR digests than on the control peptone, and exhibited strain- or species- specific peptide  
27 preferences, as evidenced by principal component analysis. Bifidobacteria generally consumed a greater  
28 amount of peptides than lactobacilli, in terms of both the mean peptide consumption and the number of  
29 peptides consumed. For bifidobacteria, peptide preferences were very diverse, but a core of 10 peptides with  
30 4 or 5 residues were consumed by all the strains. Lactobacilli behaved more homogeneously and consumed  
31 nearly only the same 6 peptides, mostly dipeptides. The peptide preferences of the different groups of  
32 bifidobacteria and lactobacilli could not be ascribed to features such as the length of the peptide or the  
33 abundance of residues with peculiar properties (hydrophobicity, polarity, charge) and likely depend on  
34 specific proteases and/or peptide transporters preferentially recognizing specific sequence motifs. The  
35 cultures of human colonic microbiota confirmed that PR digest promoted the growth of commensal  
36 bifidobacteria. This study demonstrated that peptides derived from simulated gastrointestinal digestion of PR  
37 supported the growth of most lactobacilli and bifidobacteria.

38

39

40 **Keywords**

41 Parmigiano Reggiano, Simulated Gastrointestinal Digestion, *Bifidobacterium*, *Lactobacillus*, Intestinal  
42 Microbiota, Bioactive Peptides, Cheese.

43

44

45 **Abbreviations**

46 **PR**, Parmigiano Reggiano; **GIT**, gastrointestinal tract; **SLAB**, starter lactic acid bacteria; **NSLAB**, non-  
47 starter lactic acid bacteria; **PCA**, principal component analysis; **HPLC**, High Performance Liquid  
48 Chromatography; **UPLC**, Ultra Performance Liquid Chromatography; **ESI**, Electrospray Ionization; **MS**,  
49 mass spectrometry.

50

51

## 52 **1 Introduction**

53 Parmigiano Reggiano (PR) is a raw-milk, hard cooked, long-ripened cheese of high quality and  
54 nutritional value, produced in a restricted area in northern Italy. PR is produced from bovine milk with rennet  
55 and natural whey starter and a Protected Designation of Origin, in compliance with the European norm  
56 currently in force (European Commission, 2009, 2011). The curd is heated at 55°C to select thermophilic  
57 bacterial strains and it is ripened at least for 12 months, even if much longer ageing times are usually  
58 adopted. In mature PR, the moisture is 28 to 35%. The dry weight is mostly composed of proteins and lipids  
59 fractions, the ratio of fat to protein being around 0.94, depending on milk characteristics. Ripened PR is  
60 lactose- and galactose-free and rich in free organic acids, mostly derived from bacterial fermentations, such  
61 as lactic acid (1.5 g per 100 g of PR), citric (50 mg), acetic (100 mg), propionic (0.5 mg), and butyric (120  
62 mg) acids (Gatti et al., 2014). The overall mineral content of PR is 4.0 to 4.5%, with NaCl at approximately  
63 1.5% (Gatti et al., 2014).

64 Long ripening times allow for cheese extensive proteolysis (Fox and McSweeney, 1998;  
65 McSweeney, 2004; Visser, 1993) due to the residual rennet activity and the enzymes of starter (SLAB) and  
66 non-starter (NSLAB) lactic acid bacteria. During ripening, proteolysis gives rise to the continuous evolution  
67 of oligopeptides and to the release of free amino acids (15 to 25% of protein content), while non-proteolytic  
68 aminoacyl derivatives also accumulate (Sforza et al., 2009, 2012). Casein breakdown contributes to  
69 improved digestibility, reduced allergenicity, and flavor development (Alessandri et al., 2012; Sforza et al.,  
70 2012). The potential biological activity of PR peptides has recently attracted particular interest (Korhonen et  
71 al., 2009; Tidona et al., 2009). *In vitro* studies revealed calcium binding properties (Kim et al., 2004; Pinto et  
72 al., 2012) and antioxidant (Bottesini et al. 2013; Gupta et al., 2009), antihypertensive (Bernabucci et al.,  
73 2014), antimicrobial activities (Benkerroum, 2010; Rizzello et al., 2005)..

74 The nutritional value of PR, coupled with the potential beneficial properties of bioactive peptides  
75 that could impact health, suggested its use as functional food in a dietary therapy for subjects with  
76 inflammatory GIT diseases resulting from alimentary intolerance, post-therapeutic antibiotic-associated

77 dismicrobism, or post-infective conditions (Olivi et al., 1979; Pancaldi et al., 2008). In particular, a  
78 homemade food based on PR cheese was developed and successfully utilized to feed infants suffering from  
79 different forms of intestinal problems, with a rapid clinical improvement and normalization of the gut. The  
80 high digestibility and the high amounts of short chain fatty acids, amino acids, and oligopeptides easily  
81 absorbed in the bowel likely support this beneficial effect. Furthermore, the hypoallergenicity, the absence of  
82 lactose, and the high sodium content that restores losses of salts and reduces the secretions of intestinal cells  
83 have been claimed as responsible of the positive outcome of this dietary therapy (Olivi et al., 1979; Pancaldi  
84 et al., 2008).

85         The human colon is colonized by a dense and complex bacterial community exerting important  
86 effects on the health status (Sekirov et al., 2010), wherein the commensal bifidobacteria and lactobacilli are  
87 recognized as beneficial and are commonly utilized as probiotics (Rossi and Amaretti, 2010; Walter, 2008).  
88 Previous studies demonstrated that the hydrolysates of proteins from different origins, and particularly from  
89 milk, can act as growth factors for lactobacilli or bifidobacteria (Poch and Bezkorovainy, 1991, Ibrahim and  
90 Bezkorovainy, 1994; Liepke et al., 2002; Oda et al., 2013; Meli et al., 2013, 2014). Moreover, the peptides  
91 released with the gastric digestion of an aged cheese were demonstrated to modulate the composition of mice  
92 microbiota *in vitro*, improving the abundance of bifidobacteria (Condezo-Hoyos et al., 2016). On this basis it  
93 has been hypothesized that PR peptides could promote growth and/or activity of these beneficial microbes,  
94 thus modulating the resident microbiota towards a balanced and healthy composition (Sekirov et al., 2010).

95         The health effects of PR bioactive peptides on intestinal bacteria require that they resist  
96 gastrointestinal digestion and, at least partially, reach the colon. The present study aimed to characterize the  
97 peptides derived from simulated gastrointestinal digestion of PR cheese, utilizing an *in vitro* digestion model  
98 that recently achieved an international consensus (Minekus et al., 2014). The resulting peptide mixtures were  
99 characterized by liquid chromatography coupled with mass spectrometry. In order to verify whether peptides  
100 specifically promoting the growth of beneficial bacteria and potential probiotics could be identified, the  
101 mixtures were tested as nitrogen source for bifidobacteria and lactobacilli in pure and fecal cultures.



102

## 103 **2 Materials and Methods**

### 104 *2.1 Cheese water soluble extract*

105 Samples of PR aged 16, 24, and 36 months (hereinafter referred to as PR16, PR24, and PR36,  
106 respectively) were provided by Consorzio del Formaggio Parmigiano Reggiano (Modena, Italy). Five grams  
107 of finely ground cheese were added with 45 mL of HCl 0.1 M and 1.25 mL of an aqueous solution of  
108 phenylalanyl-phenylalanine (Phe-Phe, 1 mM), that was used as internal standard. Samples were  
109 homogenized for 1 min using an UltraTurrax (IKA T50 basic, Staufen, Germany) at 4000 min<sup>-1</sup>. Insoluble  
110 proteins were removed by acid precipitation followed by centrifugation (45 min at 4°C at 3220 × g),  
111 according to Hernández-Ledesma et al. (2012). The supernatant was filtered through paper filter and  
112 extracted three times with diethyl ether to remove fats. Ether residues were removed with a rotavapor, and  
113 the residual solution was filtered subsequently through 5 µm and 0.45 µm filters.

114

### 115 *2.1 Simulated gastrointestinal digestion of PR samples*

116 All the chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) unless otherwise  
117 stated. The cheese was finely grounded and digested following the procedure, consisting in three main steps:  
118 salivary phase, gastric phase, and intestinal phase (Minekus et al., 2014). A sample of 25 g cheese was added  
119 to 17.5 mL of salivary buffer (15.1 mM KCl, 3.7 mM KH<sub>2</sub>PO<sub>4</sub>, 13.6 mM NaHCO<sub>3</sub>, 0.15 mM MgCl<sub>2</sub>, and  
120 0.06 mM (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>), 2.5 mL of 1500 U/mL amylase, 125 µL of 300 mM CaCl<sub>2</sub>, and 4.875 mL of distilled  
121 water (ratio cheese:digestive fluid of 1:1, w:v). The sample was vortexed and incubated for 2 min at 37°C on  
122 a reciprocating shaker. Then, 37.5 mL of gastric buffer (6.9 mM KCl, 0.9 mM KH<sub>2</sub>PO<sub>4</sub>, 25 mM NaHCO<sub>3</sub>,  
123 47.2 mM NaCl, 0.1 mM MgCl<sub>2</sub>, and 0.5 mM (NH<sub>4</sub>)<sub>2</sub>CO<sub>3</sub>), 8 mL of 25000 U/mL pepsin, 25 µL of 300 mM  
124 CaCl<sub>2</sub>, 1 mL of 1 M HCl and 3.475 mL of water were added (final ratio cheese:digestive fluids 1:3, w:v).  
125 The pH was adjusted to 3 with 1 M HCl. The mixture was vortexed and incubated for 2 h at 37°C on a  
126 reciprocating shaker. Finally, 55 mL of intestinal buffer (6.8 mM KCl, 0.8 mM KH<sub>2</sub>PO<sub>4</sub>, 85 mM NaHCO<sub>3</sub>,

127 38.4 mM NaCl, 0.33 mM MgCl<sub>2</sub>), 25 mL of 800 U/mL pancreatin, 12.5 mL of 75 mg/mL bile solution, 200  
128  $\mu$ L of 300 mM CaCl<sub>2</sub>, 750  $\mu$ L of 1 M NaOH and 6.55 mL of distilled water were added (final ratio  
129 cheese:digestive fluids 1:7, w:v). The pH was adjusted to 7 using 1 M NaOH. The sample was vortexed and  
130 incubated for 2 hours at 37°C on a reciprocating shaker. To stop the digestion the sample was heated at 95°C  
131 for 15 min, then cooled. Since the assessment of digestion is strongly affected by the analysis of the digested  
132 fraction, the samples were centrifuged for 45 min at 4°C at 3220  $\times$  g to remove insoluble proteins and  
133 undigested components (Minekus et al., 2014). The supernatant was filtered through 0.45  $\mu$ m membranes.  
134 For chromatographic analysis, 196  $\mu$ L of sample was supplemented with 4  $\mu$ L of 1 mM Phe-Phe.

135

## 136 2.2 HPLC-ESI-MS/MS analysis.

137 In order to identify the peptides, the samples were separated by a reverse phase column (Jupiter 5  $\mu$ m  
138 C18, 90 Å, 2  $\times$  250 mm, Phenomenex, Torrance, CA, USA) in a HPLC (High Performance Liquid  
139 Chromatography) system coupled with electrospray ionization source (ESI) and mass spectrometry (MS)  
140 tandem detector (Alliance 2695 HPLC device with triple quadrupole Quattro micro MS, Waters, Milford,  
141 MA, USA). Elution was performed with the following gradient of eluent A (water with 0.1% formic acid and  
142 0.2% acetonitrile) and eluent B (acetonitrile with 0.1% formic acid): 0-12 min 100% A, 12-77 min from  
143 100% A to 50% A, 77-81 min 50% A, 81-82 min from 50% A to 0% A, 82-90 min 0% A, 90-91 min from  
144 0% A to 100% A, 91-110 min 100% A. The samples were first analyzed in Full Scan mode, to identify the  
145 characteristic ions and the retention time of the unknown compounds, and then in Daughters Scan modality  
146 using a variable collision energy (CE = 10, 20, and 30 eV). HPLC-ESI-MS/MS parameters were: flow 0.2  
147 mL/min; analysis time 110 min; column temperature 35°C; injection volume 40  $\mu$ L; acquisition time 0-92  
148 min for the water soluble extracts and 7-90 min for the digested samples; ionization type: positive ion mode;  
149 scan range 70-2000  $m/z$  (interfering compounds bias was excluded by comparison with a scan range of 100-  
150 2000  $m/z$ ); capillary voltage 3.2 kV; cone voltage 30 V; source temperature 100°C; desolvation temperature  
151 150°C; cone gas flow 100 L/h; desolvation gas flow 650 L/h.

152 Peptides were manually identified as follows. Molecular ions were recorded from the Full Scan  
153 chromatogram, then they were fragmented in Daughter Scan modality using the triple quadrupole equipment.  
154 The MW of each peptide was launched in FindPept tool (<http://web.expasy.org/findpept> last accessed on  
155 September 2016) against the main milk proteins ( $\alpha$ -casein,  $\beta$ -casein,  $\kappa$ -casein,  $\alpha$ -lactalbumin and  $\beta$ -  
156 lactoglobulin), to obtain a list of peptide sequences with a compatible MW. The theoretical fragmentation  
157 pattern of these sequences was determined  
158 (<http://db.systemsbio.net/proteomicsToolkit/FragIonServlet.html> last accessed on September 2016) and  
159 compared with the MS/MS spectra. The peptide sequences were assigned on the basis of the matching  
160 fragments.

161

### 162 *2.3 UPLC/ESI-MS analysis.*

163 Absolute quantification of all the peptides identified was not possible due to the unavailability of all  
164 the peptide standards, and a direct comparison of the peptide areas would have been inaccurate due to  
165 differences in ionization efficiency. Once the sequences were assigned, semi-quantitative analysis was  
166 performed using Phe-Phe as internal standard. The ratio between the chromatographic peak of the peptide  
167 and that of Phe-Phe did not yield absolute peptide concentration but allowed the comparison of the same  
168 peptide in different samples. For the semi-quantitative analysis, UPLC (Ultra Performance Liquid  
169 Chromatography) was chosen instead of HPLC, given the higher chromatographic resolution. Samples were  
170 separated by a reverse phase column (Acquity UPLC BEH 300 C18, 1.7  $\mu$ m, 2.1  $\times$  150 mm equipped with a  
171 Acquity UPLC BEH C18 VanGuard Pre-column, 300 $\text{\AA}$ , 1.7  $\mu$ m, 2.1  $\times$  5 mm, Waters) in an UPLC system  
172 coupled with ESI and MS (UPLC Acquity with a single quadrupole detector SQD, Waters). In order to  
173 replicate a chromatographic trace similar to HPLC, but with shorter run time, gradient elution with the same  
174 eluents was programmed as follows: 0-7 min, 100% A; 7-50 min, from 100% A to 50% A; 50-52.6 min, 50%  
175 A; 52.6-53 min, from 50% A to 0% A; 53-58.2 min, 0% A; 58.2-59 min, from 0% A to 100% A; 59-72 min,  
176 100% A. The analysis parameters were: flow 0.2 mL/min; analysis time 72 min; column temperature 35°C;

177 sample temperature 18°C; injection volume 10 µL for water soluble extracts and 2 µL for digested samples;  
178 acquisition time 0-58.2 min for water soluble extracts and 7-58.2 min for digested samples; ionization type:  
179 positive ion mode; capillary voltage 3.2 kV; cone voltage 30 V; source temperature 150°C; desolvation  
180 temperature 350°C; cone gas flow 100 L/h; desolvation gas flow 650 L/h. Samples were analyzed in the Full  
181 Scan mode, with a scan range of 100-2000 *m/z*. The ions of interest were integrated using MassLynx  
182 software (4.0) and semi-quantified using Phe-Phe area in order to compare relative peptide intensities.

183

#### 184 *2.4 Strains and culture conditions*

185 *Bifidobacterium* strains were obtained from the collection of the Dept. of Life Sciences (University  
186 of Modena and Reggio Emilia, Italy), the Scardovi Collection of Bifidobacteria (Buscob, University of  
187 Bologna, Italy), or the American Type Culture Collection (ATCC, USA). *Lactobacillus* strains were obtained  
188 from the collection of Dept. of Food Science (University of Parma, Italy). Bifidobacteria and lactobacilli  
189 were routinely cultured anaerobically at 37°C in Lactobacilli MRS broth (BD Difco, Sparks, NV, USA). For  
190 culturing bifidobacteria, MRS broth was supplemented with 0.5 g/L cysteine hydrochloride.

191

#### 192 *2.5 Pure culture conditions*

193 Utilization of PR digests was investigated in a basal medium (hereinafter referred to as BM) where  
194 PR16, PR24, PR36 digests or Bacto peptone (BD Difco) were utilized as nitrogen source, the latter as  
195 positive control. The medium contained lactose, 10 g/L; PR hydrolysates or Bacto peptone (BD Difco), 5  
196 g/L; ammonium citrate, 2 g/L; yeast nitrogen base w/o amino acids and ammonium sulfate (BD Difco), 1.7  
197 g/L; polysorbate 80, 1 g/L; sodium acetate trihydrate, 5 g/L; K<sub>2</sub>HPO<sub>4</sub>, 2 g/l; MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.1 g/l; MnSO<sub>4</sub> ·  
198 H<sub>2</sub>O, 0.05 g/L; cysteine hydrochloride, 0.5 g/l; pH was corrected to 6.5. Bacteria inoculated (5% v/v) in BM  
199 based media were incubated 24 h at 37°C, and passaged at least three times before evaluation of growth and  
200 peptide consumption.

201 Growth of bifidobacteria was determined in triplicate by measuring the turbidity at 600 nm (OD<sub>600</sub>)

202 at the beginning and after 24 h of incubation. Growth of lactobacilli was evaluated in triplicate using a  
203 BacTrac 4300 Microbiological Analyzer system (Sylab, Neupurkersdorf, Austria). The BM culture was  
204 diluted 1:100 in Ringer's solution (Oxoid, Basingstoke, UK), then 0.1 ml were used to inoculate tubes  
205 containing 6 mL of BM media. The impedance measurement was performed at 37°C. Bacteria growth was  
206 measured as E%, where E-value is the impedance change revealed by the system at the electrode surface.  
207 Variation in electrical conductivity, monitored during time, is proportional to the change in the number of  
208 microorganisms (Bancalari et al., 2016). Maximum value of E% was recorded as index of maximum  
209 microbial growth.

210

## 211 *2.6 Cultures of intestinal microbiota*

212 Batch cultures of fecal microbiota were performed in bioreactors containing 200 mL of FM medium,  
213 developed from the media described by Walker et al. (2005) and Duncan et al. (2002), with some  
214 modifications: beech wood xylan, 0.6 g/L; citrus pectin, 0.6 g/L; maize amylopectin, 0.6 g/L; larch wood  
215 arabinogalactan, 0.6 g/L; potato starch, 3 g/L; fructans (Synergy1, Beneo, Mannheim, Germany), 3 g/L;  
216 peptone, 5 g/L; ammonium citrate, 2 g/L;  $\text{KH}_2\text{PO}_4$ , 2 g/L; NaCl, 4.5 g/L;  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  0.5 g/L;  $\text{CaCl}_2 \cdot$   
217  $2\text{H}_2\text{O}$  0.045 g/L;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 0.005 g/L; hemin, 0.01 g/L; bile salts (Oxgall, BD Difco) 0.05 g/L,  
218 resazurin, 0.6 mg/L; reducing solution, 40 ml/L; mineral solution, 2 ml/L; vitamin solution, 1.4 ml/L.  
219 Reducing solution contained: cysteine hydrochloride, 12.5 g/L;  $\text{NaHCO}_3$ , 80 g/L. Mineral solution  
220 contained: EDTA, 500 mg/L;  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ , 200 mg/L;  $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ , 10 mg/L;  $\text{MnCl}_2 \cdot 7\text{H}_2\text{O}$ , 3 mg/L;  
221  $\text{H}_3\text{BO}_3$ , 30 mg/L;  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ , 20 mg/L;  $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ , 1 mg/L;  $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 2 mg/L;  $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ , 3  
222 mg/L. Vitamin solution contained: menadione, 1 g/L; biotin 2 g/L; calcium pantothenate 2 g/L; nicotinamide,  
223 10 g/L; vitamin B12, 0.5 g/L; folic acid 0.5 g/L; thiamine, 4 g/L; PABA, 5 g/L. Reducing, mineral and  
224 vitamin solutions were filter sterilized and added to the autoclaved medium. PR24 digest or Bacto peptone  
225 were utilized as nitrogen source at the concentration of 5 g/L in parallel batches, carried out in duplicate. The  
226 inoculum was prepared pooling equal amounts of fecal microbiota from three healthy adult subjects. The

227 feces were homogenized (5% w/v) with the medium in the anaerobic cabinet (Anaerobic System, Forma  
228 Scientific, Marietta, OH) under a 85% N<sub>2</sub>, 10% CO<sub>2</sub>, 5% H<sub>2</sub> atmosphere. 10 mL of the suspension were  
229 inoculated into bioreactors (Sixfors V3.01, Infors, Bottmingen, Switzerland) each containing 190 mL of  
230 fresh sterile medium, and the processes proceeded for 48 h (37°C, CO<sub>2</sub> atmosphere, pH 6.2, stirring 60 rpm).  
231 5 mL samples were collected at 0, 6, 12, 24 h and stored at -20°C until analyzed.

232 The abundance of total eubacteria, bifidobacteria and lactobacilli was determined by qPCR  
233 with the following primer pairs: F-Eub/R-Eub (TCCTACGGGAGGCAGCAGT/  
234 GGACTACCAGGGTATCTAATCCTGTT) (Nadkarni et al., 2002), BiTOT-F/BiTOT-R  
235 (TCGCGTCYGGTGTGAAAG/CCACATCCAGCRTCCAC) (Rinttilä et al., 2004), and Lac-F/Lac-R  
236 (GCAGCAGTAGGGAATCTTCCA/GCATTYCACCGCTACACATG) (Castillo et al., 2006). DNA  
237 extraction and amplification reactions were performed according to Simone et al. (2014). The following  
238 thermocycle was utilized for eubacteria and lactobacilli: 98°C for 2 min; 40 cycles of 98°C for 5 s, 64.5°C  
239 for 5 s, and 95°C for 1 min; 65°C for 1 min. The same program was utilized for bifidobacteria, with  
240 annealing temperature of 62.5°C.

241

## 242 *2.7 Principal component analysis of peptide consumption*

243 Principal component analysis (PCA) (Massard et al., 1997; Wold et al., 1987) was carried out to  
244 compare the peptide consumption by bifidobacteria and lactobacilli. Due to matrix effects resulting from  
245 components of the medium, consumption could be evaluated only for 63 out of the 72 peptides occurring in  
246 the extracts. The experimental data were analyzed using PLS Toolbox software ver. 8.1.1 (Eigenvector  
247 Research Inc., Wenatchee, WA, USA) for the calculation of PCA models. A PCA model was calculated on a  
248 dataset with size {45, 63} including all the 45 samples (i.e. 15 strains × 3 ripening ages of PR) obtained by  
249 digestions carried out with bifidobacteria and lactobacilli, each one characterized by the consumption values  
250 of 63 peptides. Two further PCA models were calculated considering bifidobacteria and lactobacilli  
251 separately, i.e. on two separate datasets with size equal to {24, 63} and to {21, 63}, respectively. Before

252 calculating the PCA models, the variables were pre-processed using mean centering. The number of  
253 significant PCs, i.e., the number of PCs bearing useful information, was selected based on the scree plot  
254 reporting the percentage of variance explained by each PC vs. the PC number.

255

### 256 **3 Results**

#### 257 *3.1 Effects of simulated digestion on the peptide profile of PR*

258 The peptide profile of PR16, PR24, and PR36 was characterized by HPLC/ESI-MS/MS before and  
259 after the *in vitro* simulated gastrointestinal digestion (Figure 1A, 1B). Intact caseins are not taken into  
260 account, since they were removed from both the water soluble extracts and from the digested samples.  
261 Before the simulated digestion, a common peptide profile was detected in the water extracts of PR16, PR24,  
262 and PR36 samples (Fig. 1A). 63 main peptides were identified, including some N-lactosyl amino acids, with  
263 a length up to 103 residues and a molecular weight spread over a wide range (Supporting Information: Table  
264 S1). 31 peptides had a MW < 500 Da, 2 had a MW of 500-1000 Da, 12 fell in the range 1000-2000 Da.  
265 Longer peptides were also identified, 12 with MW of 2-5 kDa and at 6 with MW of 5-20 kDa. Moreover, the  
266 two isoforms of  $\beta$ -lactoglobulin (A and B) were clearly detectable at 40.14 min.

267 After *in vitro* digestion, the mixtures of PR16, PR24, and PR36 still shared similar profile. The  
268 digests lacked intact proteins, including  $\beta$ -lactoglobulins, and 61 out of the 63 peptides identified in water  
269 soluble extracts were no longer detectable, being degraded into shorter ones (Fig. 1B). The sole exception  
270 were FVAPFPEVFGK and the phosphopeptide LEELNVPGEIVESLSSS. The former was present in the  
271 water soluble extract, especially of the PR36, and remained in similar amounts after digestion, whereas the  
272 latter occurred in the digests in minor amount. N-lactosyl amino acids also resisted digestion, but their  
273 chromatographic signal was negligible, compared with those of peptides.

274 As a whole, 72 main peptides were identified in the PR digests (Supporting Information: Table S2)  
275 and, with the exception of P3, P13, P71, and P72, were assigned to specific sequences. Compared with the  
276 undigested water extracts, molecular weight distribution of the peptides was shifted toward lower MW: 12

277 peptides had MW < 500 Da, 41 had a MW of 500-1000 Da, constituting the majority of peptides of the  
278 digests, and 14 peptides had a MW of 1000-2000 Da. Only 3 peptides presented a MW > 2 kDa, none of  
279 them exceeding 2700 Da. (Fig. 1B).

280

### 281 3.2 Growth of bifidobacteria and lactobacilli on PR digests as nitrogen source

282 Twenty seven *Bifidobacterium* strains belonging to the species *B. adolescentis*, *B. animalis* subsp.  
283 *animalis*, *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*,  
284 and *B. pseudocatenulatum*, and 30 *Lactobacillus* strains belonging to the species *L. casei*, *L. paracasei* and  
285 *L. rhamnosus* were investigated for the ability to grow utilizing PR hydrolysates as nitrogen source. All the  
286 strains grew both in the control medium and the PR-based media (Fig. 2), exhibiting a species specific  
287 growth yield, with few exceptions. Within the phylogenetically related group of *B. adolescentis* and *B.*  
288 *pseudocatenulatum*, the strains belonging to the former species gave similar yields in the diverse BM media,  
289 while the strains belonging to the latter grew more abundantly with PR digests. All the strains of *B. breve*  
290 and most of *B. longum* grew more abundantly in the media containing the PR digests than in the control. On  
291 the contrary, most *B. bifidum* and *B. animalis* grew more abundantly using the control peptone as nitrogen  
292 source than PR digests. *L. casei* grew more abundantly on PR digests, while *L. paracasei* 2306 and the *L.*  
293 *rhamnosus* strains 2323, 2303, 2302, 2409 grew better on the control peptone. A trend common to all the  
294 tested species and strains, with respect to the growth and the age of PR, could not be found. However, most  
295 of the bifidobacteria that preferred PR digests over the control peptone grew more abundantly on PR36 or  
296 PR24 than on PR16. On the other hand, most of the lactobacilli preferring PR digests over the peptone, grew  
297 better on PR16 or PR24 than on PR36.

298

### 299 3.3 Consumption of PR peptides by bifidobacteria and lactobacilli

300 Peptide utilization was analyzed by UPLC/ESI-MS for 8 *Bifidobacterium* and 7 *Lactobacillus*  
301 strains, representative of different species/subspecies and selected among those with best growth on PR



302 digests, (Fig. 3). Peptide profiles of cultures grown for 24 h on BM medium supplemented with PR16, PR24,  
303 or PR36 digests were compared with the profiles of non-inoculated media incubated under the same  
304 conditions. The percentage of consumption of each peptide was determined (Fig. 3) and the consumption  
305 profiles were compared by PCA (Fig. 4).

306 Bifidobacteria consumed a greater number of peptides and at a greater extent than lactobacilli (Fig.  
307 3). Thus, bifidobacteria and lactobacilli clustered separately in the score plot of the first 2 PCs, which  
308 explains 66% of total data variance (Fig 4a). Lactobacilli are closely grouped, while bifidobacteria are much  
309 more dispersed, indicating that lactobacilli behaved more homogenously than bifidobacteria with respect to  
310 peptide utilization. Based on the corresponding loading plot (Fig. 4b), consumption of peptides P4, P9, P12,  
311 P18, P20, P48, P49, P68, and P72 characterized the behavior of lactobacilli, whereas peptide consumption  
312 was more diversified among bifidobacteria.

313 Bifidobacteria presented different patterns of utilization (Fig. 3). All the strains nearly depleted P1,  
314 P27, P28, P30, and P45 while they did not consume, or scarcely consumed, P39, P53, P69, and P70.  
315 However, preferential utilization or non-utilization of most peptides was associated to one or a group of  
316 strains, as revealed by the PCA model calculated on bifidobacteria (Fig. 5a, 5b). The score plot of the first 2  
317 PCs (accounting for 64% of total data variance) shows that bifidobacteria are clearly grouped (Fig. 5a) on the  
318 basis of their peptide preferences observable in the corresponding loading plot (Fig. 5b). *B. adolescentis*  
319 RBB4 (B1), located distant from other bifidobacteria along PC1 (Fig. 5a), was the most efficient in utilizing  
320 PR peptides. It consumed 45-47 peptides for more than 75% and 21-23 for more than 95% (Fig. 3).  
321 Moreover, it was the sole strain able to consume specific peptides, such as P14, P25, P29, P47, P48, P66,  
322 P67, P68, including the diphosphopeptide P44 (Fig. 3).

323 *B. animalis* subsp. *animalis* ATCC 27536 (B3) and *B. animalis* subsp. *lactis* WC 0413 (B4) were the  
324 less efficient and grouped together in the PC1-PC2 score plot (Fig. 5A). They consumed only 8-10 peptides  
325 for more than 75%, none of them for more than 95% (Fig. 3). The strains of *B. animalis* strains consumed  
326 P33, while utilized less efficiently several peptides generally consumed by the other bifidobacteria (e.g. P12,

327 P15, P16, P19, P24, and P38) (Fig. 3).

328 *B. bifidum* B2091 (B5) and *B. breve* WC 0423 (B6) behaved similarly and differed from the other  
329 bifidobacteria along PC3 (PC3 and PC4 account for further 21% of total data variance), mostly because of  
330 greater consumption of P12, P18, P26, P33, P35, P36, P38, P57, and P71 (Fig. 3, Supporting Information:  
331 Fig. S1).

332 *B. longum* strains (B7 and B8) shared a similar behavior towards many peptides, but *B. longum*  
333 subsp. *infantis* WC 0438 (B7) was able to consume also P2, P7, P23, P52, P57, P59, P62, P63, P64, and P71.  
334 This explains the strains not grouping together, with a clear separation along PC1, PC2, (Fig. 5a) and PC4  
335 (Supporting Information: Fig. S1). Peptide consumption by *B. longum* subsp. *longum* WC 0438 (B8) and *B.*  
336 *pseudocatenulatum* WC 0403 (B2) was very similar, being largely overlapped in the first three principal  
337 components and slightly differentiating only along PC4 (Supporting Information: Fig. S1).

338 Lactobacilli were less performing in PR peptide consumption compared to bifidobacteria (Fig. 3).  
339 They all consumed P1, P4, P9, P12, P18, and P20, but generally did not use, or used at lower extent, the vast  
340 majority of the other peptides. The first 2 PCs of the PCA model calculated on lactobacilli (accounting for  
341 61% of total data variance) showed that the pattern of peptide consumption was homogenous for most of the  
342 lactobacilli, with minor differences. The major differences were observed for *L. casei* 2414 (L6) and *L.*  
343 *paracasei* 2306 (L7) (Fig. 5c) that clustered separately along PC1 and were characterized by high  
344 consumption of P15, P46, P49, P57, and P64 and by poor utilization of P13, P27, P68, and P72 (Fig. 5d).  
345 P68 and P72 were consumed for more than 60% by all the lactobacilli, with the exception of *L. casei* 2414  
346 (L6) and *L. paracasei* 2306 (L7).

347 All the other lactobacilli clustered together at high values of PC1 and presented a similar pattern of  
348 peptide utilization. *L. rhamnosus* 2232 (L1), *L. rhamnosus* 2325 (L2), *L. rhamnosus* 2362 (L3), *L. casei* 2240  
349 (L4), and *L. casei* 2404 (L5) are distributed along PC2. *L. rhamnosus* 2232 (L1) lies at positive values of  
350 PC2, while *L. rhamnosus* 2325 (L2) and *L. casei* 2404 (L4) are found at negative PC2 values (Fig. 5c). In  
351 fact, despite their pattern of utilization was very similar, *L. rhamnosus* 2232 (L1) was the most efficient in

352 peptides utilization, with 10 peptides consumed for more than 75%, and differed especially for a greater  
353 consumption of P16 and few other peptides (Fig. 3 and Fig. 5d). On the contrary, *L. rhamnosus* 2325 (L2)  
354 and *L. casei* 2404 (L5) were the least performing in PR peptides consumption, with only the most 6 peptides  
355 consumed by all the strains utilized for more than 75% (Fig. 3).

356 The score plots of both bifidobacteria and lactobacilli did not exhibit any grouping and/or  
357 distribution along the principal components, based on the extent of the growth of the bacteria. Similarly, also  
358 the loading plots of both bifidobacteria and lactobacilli did not exhibit any trend along the principal  
359 components, based on the main characteristic of the peptides, such as the length, the abundance of  
360 hydrophobic, polar, and positively/negatively charged amino acids (data not shown), or the presence of  
361 sulfur-containing residues.

362

### 363 3.4 Microbiota cultures

364 Cultures of intestinal microbiota were performed in a medium containing 5 g/L of PR24 digest as  
365 only nitrogen source or peptone as control (Fig. 6). In both the cultures, total bacteria similarly increased by  
366 more than 2 magnitudes in the first 6 h of incubation, then remained stationary at a level of approx.  $10 \text{ Log}_{10}$   
367 cells/mL (Fig. 6A). Bifidobacteria were  $6.4 \text{ Log}_{10}$  cells/mL at the beginning of batch process, accounting for  
368 4-5% of total bacteria. They grew abundantly in the first 6 h, up to  $8.7$  and  $8.4 \text{ Log}_{10}$  cells/mL in PR24 and  
369 control cultures, respectively. In control cultures bifidobacteria remained stable, representing approx. the 5%  
370 of eubacteria. In PR24 cultures, they increased up to 8% after 12 h and 14% after 24 h, even though in this  
371 latter time-point the higher abundance arose also from the slight decrease in total bacteria. Unlike  
372 bifidobacteria, lactobacilli never appeared above the limit of detection of  $4 \text{ Log}_{10}$  cells/mL. Most of the  
373 peptides of PR24 digest were consumed in the first 6 h of incubation and completely disappeared in the  
374 following incubation hours (Fig. 6B).

375

## 376 4 Discussion

377 The present study aimed to characterize the peptides that are expected to escape digestion of PR and  
378 to investigate whether they could support the growth of beneficial bacteria in the colon. The water soluble  
379 extracts of PR contained a few intact proteins (e.g. the two isoforms of  $\beta$ -lactoglobulin) and a variety of  
380 peptides of 2 to 103 residues originated during ripening through the proteolytic cleavage of cheese proteins  
381 (mainly  $\alpha$  and  $\beta$ -casein) by rennet, SLAB, and NSLAB (McSweeney, 2004). The water extracts of PR16,  
382 PR24, and PR36 shared similar profile, consistently with previous evidence that the most relevant  
383 proteolytic transformations occur in the first 12 months, before the PR can be sold on the market (Sforza et  
384 al. 2008).

385 Few previous studies performed *in vitro* digestion of PR, but mainly focused on quantification of  
386 specific peptides (Bordoni et al., 2011; De Noni and Cattaneo, 2010; Basiricò et al., 2015), without providing  
387 a thorough characterization of the whole profile. The main peptide composition of PR after simulated  
388 digestion is reported herein for the first time. The PR digests lacked serum proteins and any peptide longer  
389 than 24 residues. The new peptides derived from the hydrolysis of the high molecular weight peptides  
390 already present in cheese and from the breakdown of intact  $\alpha$ - and  $\beta$ -casein and whey proteins. Any  
391 predictable and specific cleavage sites could be found in peptide sequences, consistently with the fact that the  
392 proteolytic activity of pancreatin is due to trypsin, chymotrypsin, but also elastase, carboxypeptidase and  
393 other minor proteases, and suggesting that many peptides are generated by the action of an exopeptidase or  
394 from aspecific cleavages.

395 Some peptides identified in the digests bear sequences of biological interest. Nonetheless, it is not  
396 clear whether they could be effectively active, since the sequences were found within longer peptides for  
397 which the biological activity has not been investigated so far. In particular, the sequences of the opioids  $\beta$ -  
398 casomorphin 5 (YFPFG) and  $\beta$ -casomorphin 7 (YFPFGPI) and the ACE inhibitor peptide  $\beta$ -casomorphin 9  
399 (YFPFGPIP) were found within longer peptides originating from  $\beta$ -casein, such as P50, P56, and P58  
400 (Nguyen et al, 2015). The sequences of the ACE inhibitor tripeptides VPP and IPP were similarly found  
401 within longer peptides of the digests, such as P63, P69, and P70. The ACE inhibitor sequence  $\alpha_{s1}$ -cn f(24-

402 32) (FVAPFPEVF) was found within peptide P59. Other bioactive peptides which could occur in PR were  
403 no longer detectable after digestion, thus some doubts arise about their real availability *in vivo*.

404 Potential health promoting effects of PR peptides are supported by observation that the digests of PR  
405 sustained abundant growth of pure cultures of bifidobacteria and lactobacilli, natural beneficial colonizers of  
406 the human colon and potential probiotics (Rossi and Amaretti, 2010; Walter, 2008). Bifidobacteria were  
407 more efficient in consuming PR peptides than lactobacilli, with a greater number of peptides consumed at a  
408 greater extent. Peptide preferences of bifidobacteria were specific for each strain, as evidenced by PCA, with  
409 a core of peptides with 4 or 5 residues consumed by all strains (P1, P27, P28, P30, and P45). On the other  
410 hand, lactobacilli behaved very homogeneously and seemed to prefer the same peptides, mostly di- and  
411 tripeptides, such as P4, P9, P12, P18, and P20. The differences among lactobacilli were minor and restricted  
412 to a limited number of peptides, such as P64, P68, and P72. The lower peptides consumption by lactobacilli  
413 is consistent with the ability of NSLAB to use also free amino acids and other nitrogen compounds derived  
414 from nucleic acids and casein degradation (Kieronczyk, 2001; Sgarbi et al., 2014). Despite PCA enabled  
415 clustering bacteria on the basis of peptide utilization, no grouping and/or distribution of the peptides could be  
416 established based on their main characteristics. In fact, the peptide preferences of the different groups of  
417 bifidobacteria and lactobacilli could not be ascribed to features such as the length of the peptide, the presence  
418 of sulfur-containing amino acids, or the abundance of residues with peculiar properties (hydrophobicity,  
419 polarity, charge) and likely depend on specific proteases and/or peptide transporters preferentially  
420 recognizing specific sequence motifs in the peptides.

421 The bifidogenic effect of PR digests in pure and microbiota cultures is coherent with previous  
422 studies which described the stimulation of bifidobacteria by peptic and/or tryptic digests of certain milk  
423 proteins (Poch and Bezkorovainy, 1991; Oda et al. 2013; Condezo-Hoyos et al. 2016). In microbiota  
424 cultures, the digest of PR24 promoted the growth of bifidobacteria, the counts of which increased in the first  
425 hours of fermentation and achieved a higher relative abundance towards the end of the fermentation,  
426 compared with control cultures. However, cultivation of intestinal microbiota in PR-based medium resulted

427 in rapid and complete consumption of peptides in the first hours of incubation, confirming the intense  
428 breakdown and assimilation of peptides by the dense proteolytic population of the colon (Richardson et al.,  
429 2013). Unlike bifidobacteria, the population of lactobacilli never increased in microbiota cultures over the  
430 limit of detection of qPCR quantification (4 Log<sub>10</sub> cells/mL), likely because resident lactobacilli represent a  
431 minority population within the human gut microbiota (Quartieri et al., 2016; Rossi et al., 2016).

432

## 433 **5 Conclusions**

434 The present study describes several new, previously not reported, peptides originating from cheese  
435 digestion, thus opening the question whether they are bioactive. Determining the bioactivity of the peptides  
436 of PR digests is attractive and deserves further studies. Despite the limitation of the experimental procedure,  
437 that did not take into account the absorption in the small intestine, it was demonstrated that PR digests  
438 promoted the growth of bifidobacteria both in pure and in microbiota cultures. This observation strengthens  
439 the potential impact on the microbiota as a potential mechanism of health promotion of PR dietary  
440 supplementation, even though the highly proteolytic environment and the strong and unpredictable  
441 competition among several other bacteria make impossible to predict what can happen *in vivo*. The  
442 interaction between specific peptides and colonic bacteria is still largely unexplored and, based on the results  
443 herein presented, opens intriguing perspective in the modulation of the gut microbiota towards a healthy  
444 composition and deserves deeper investigation.

445

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448

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578 **Figure legends**

579 **Figure 1.** Overlaid Full Scan chromatograms obtained for the waters soluble extracts (**Panel A**) and the  
580 digests (**Panel B**) of PR16 (black), PR 24 (green), and PR 36 (orange). Chromatograms were obtained by  
581 UPLC/ESI-MS and they are showed as Total Ion Current (TIC); run time: 72 min.

582 **Figure 2.** Growth of 27 strains of *Bifidobacterium* (**Panel A**) and 30 of *Lactobacillus* (**Panel B**) in BM  
583 medium containing 5 g/L control peptone (grey) or PR the digests from PR16 (yellow), PR24 (orange), and  
584 PR36 (red). Growth of bifidobacteria and lactobacilli is reported as the increase of turbidity (OD<sub>600</sub>) and  
585 impedance (E%), respectively. Values are means, n = 3, SD < 0.1. The box and whiskers plots (**Panel C**)  
586 report, for each species or group of species/subspecies, the increase in the different media. Boxes indicate the  
587 median and 25<sup>th</sup> and 75<sup>th</sup> percentiles; whiskers indicate the minimum and the maximum.

588 **Figure 3.** Peptide consumption by *Bifidobacterium* and *Lactobacillus* strains cultured for 24 h in BM  
589 medium containing 5 g/L of PR16, PR24, and PR36 digests. Percentage consumption, relative to non-  
590 inoculated media similarly incubated for 24 h at 37°C, are reported as colors ranging from the lowest  
591 (deepest green) to the highest (deepest red) consumption. Values are means, n = 3, SD always < 7.

592 **Figure 4.** PCA model of peptide consumption by *Bifidobacterium* and *Lactobacillus* strains: PC1-PC2 score  
593 plot (**a**) and loading plot (**b**).

594 **Figure 5.** PCA model calculated on *Bifidobacterium* strains: PC1-PC2 score plot (**a**) and loading plot (**b**);  
595 PCA model calculated on *Lactobacillus* strains: PC1-PC2 score plot (**c**) and loading plot (**d**).

596 **Figure 6.** Cultures of human intestinal microbiota with PR24 digests. **Panel A:** time-course of total  
597 eubacteria and bifidobacteria in FM medium supplemented with 5 g/L of PR24 digests (orange) or control  
598 peptone (grey). Bacteria were quantified by qPCR. Symbols: total eubacteria, dotted line; bifidobacteria,  
599 dashed line; relative amount of bifidobacteria in the microbiota, solid line. Values are means ± SD, n = 3.

600 **Panel B:** Overlaid chromatograms of PR24 cultures of intestinal microbiota at 0 (black), 6 (green), and 12 h  
601 (yellow) of incubation. Chromatogram was obtained by UPLC/ESI-MS and they are showed as Total Ion  
602 Current (TIC); run time: 72 min.

Figure 1  
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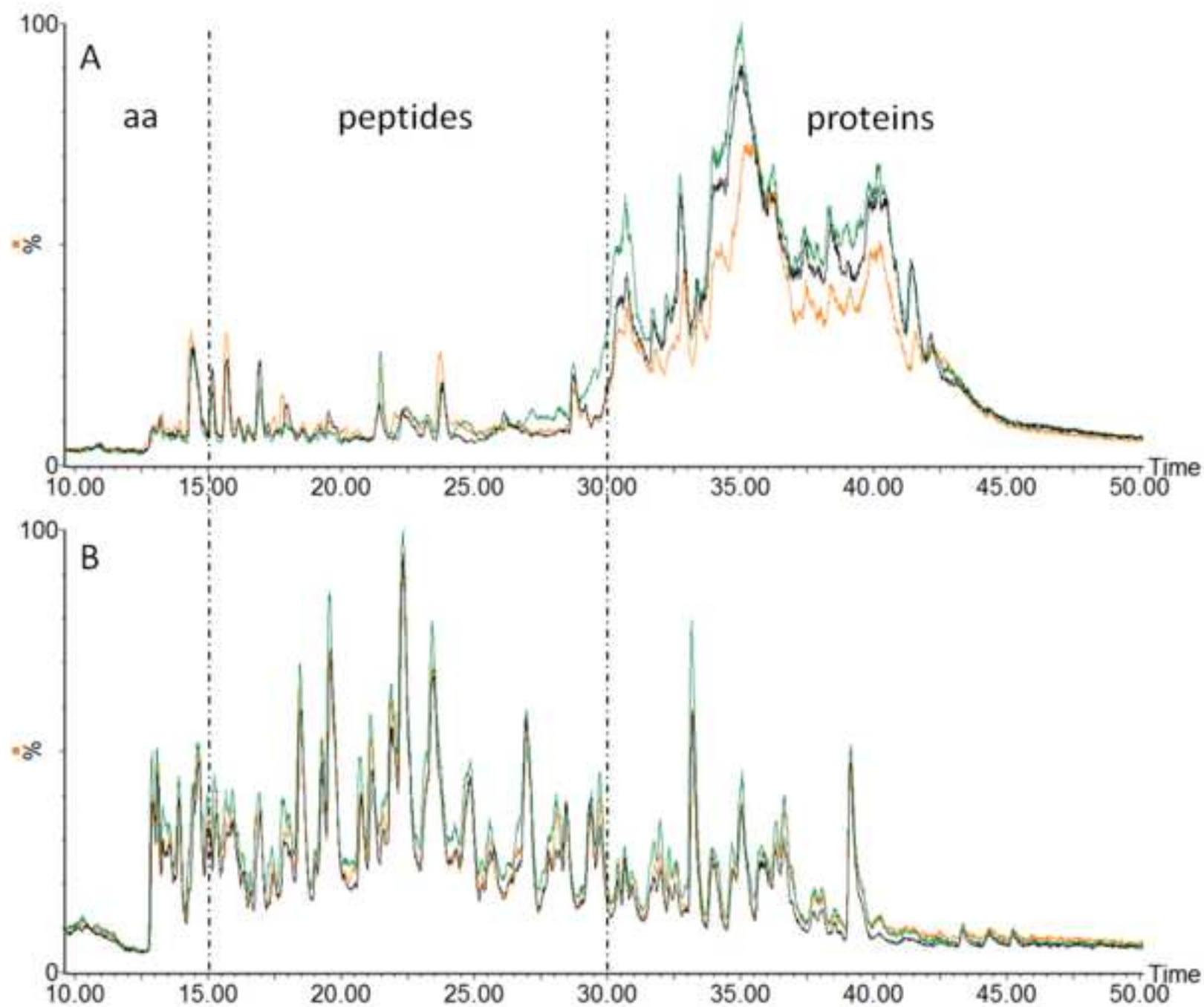


Figure 2 Revised

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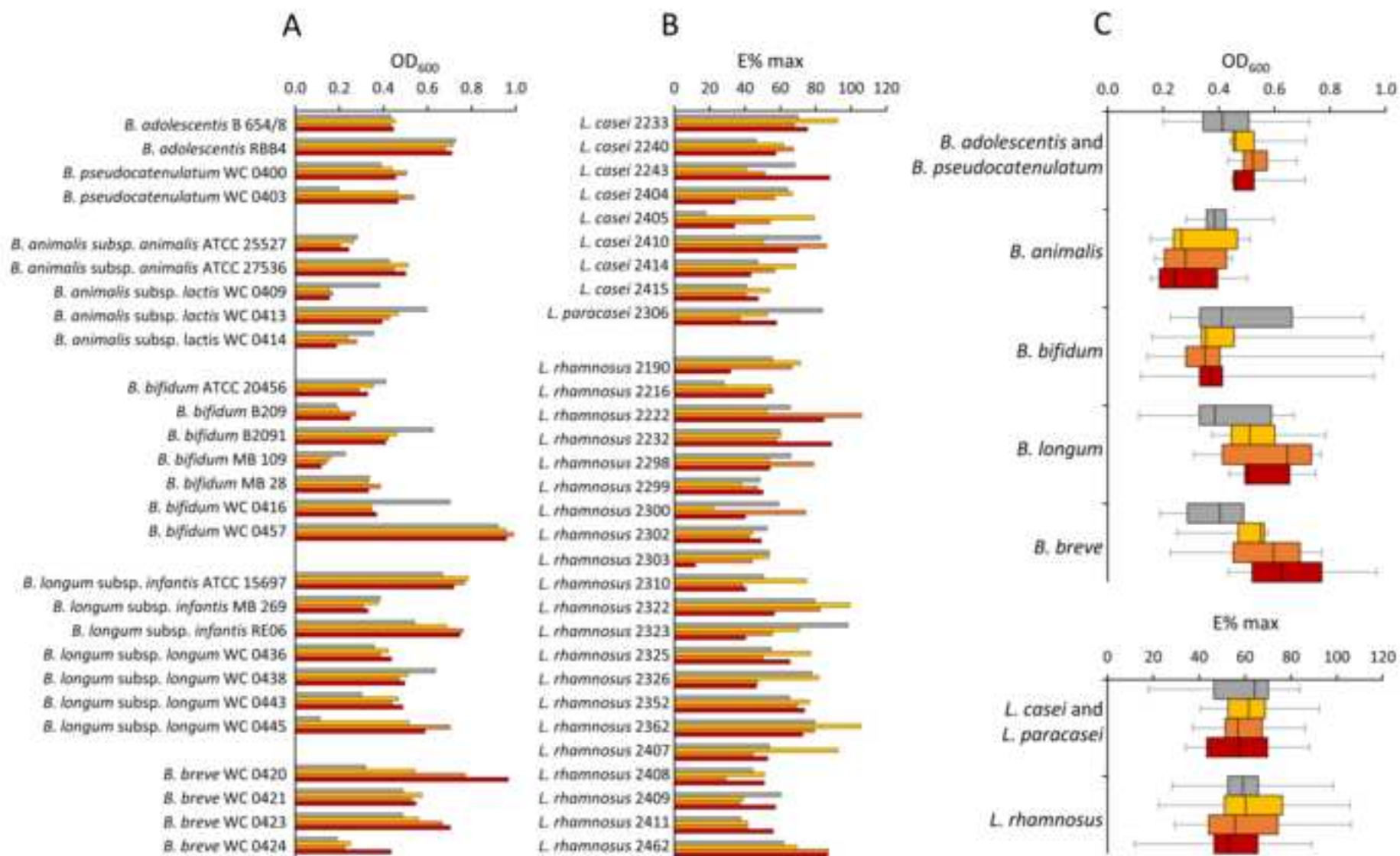


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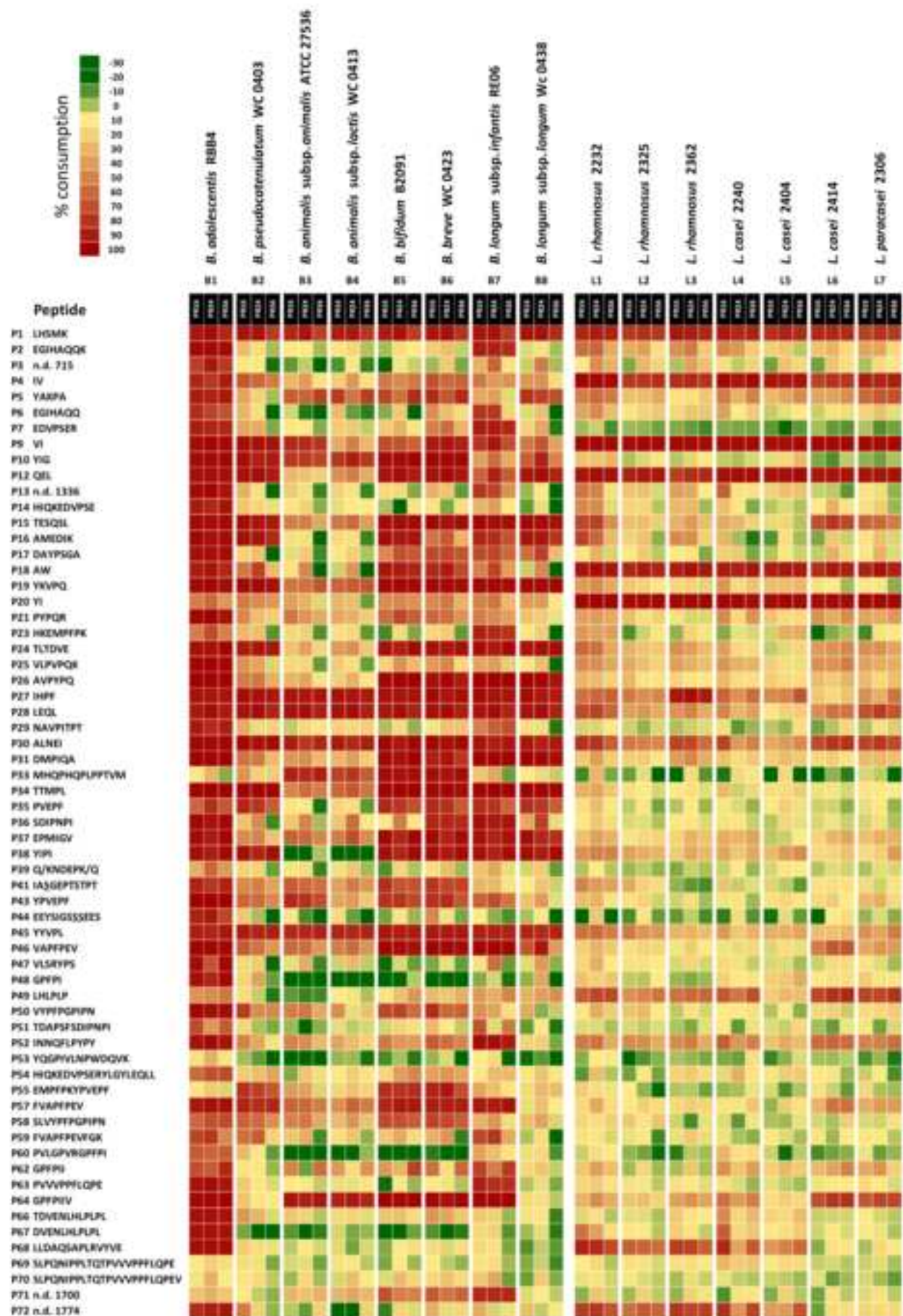




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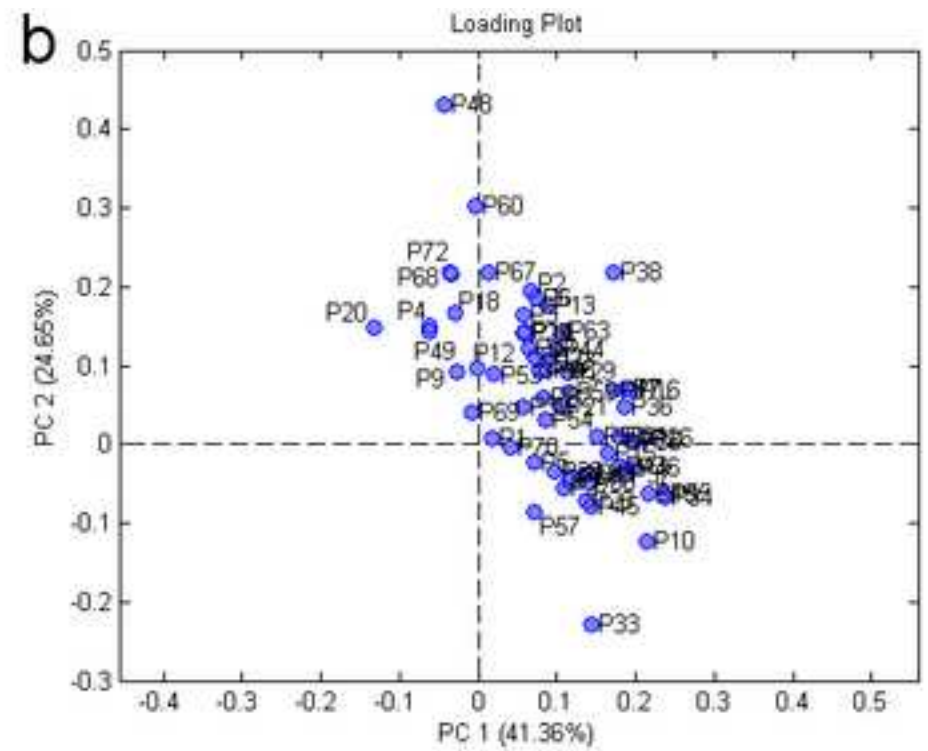
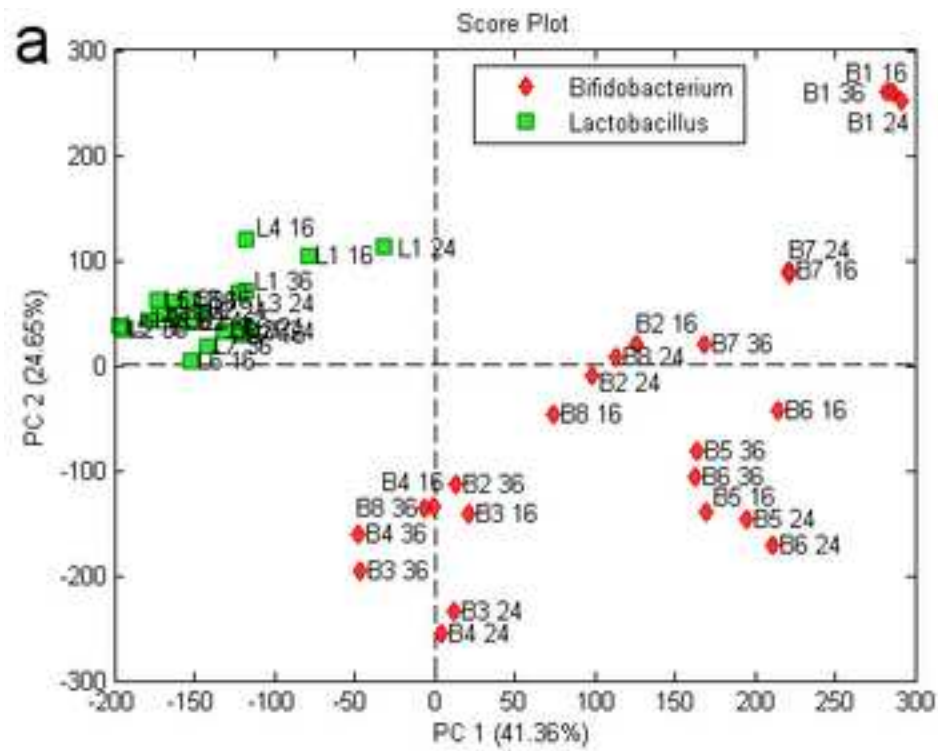


Figure 5  
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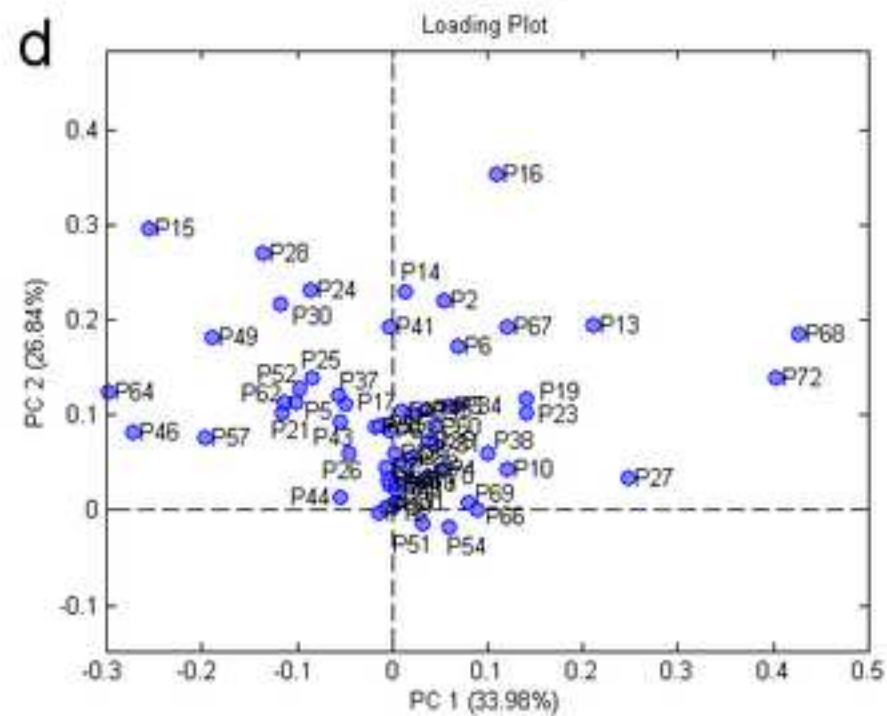
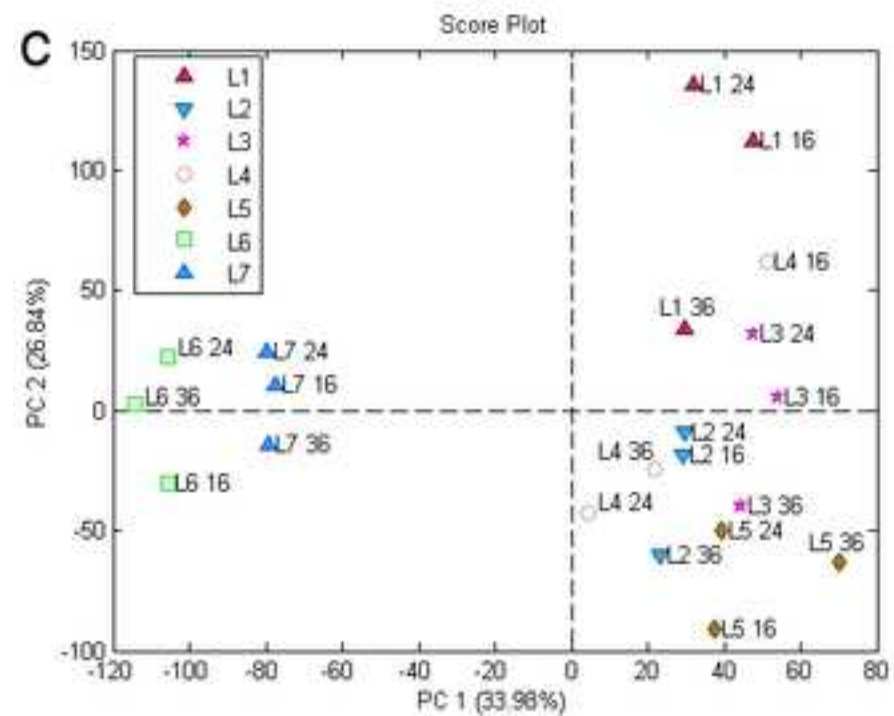
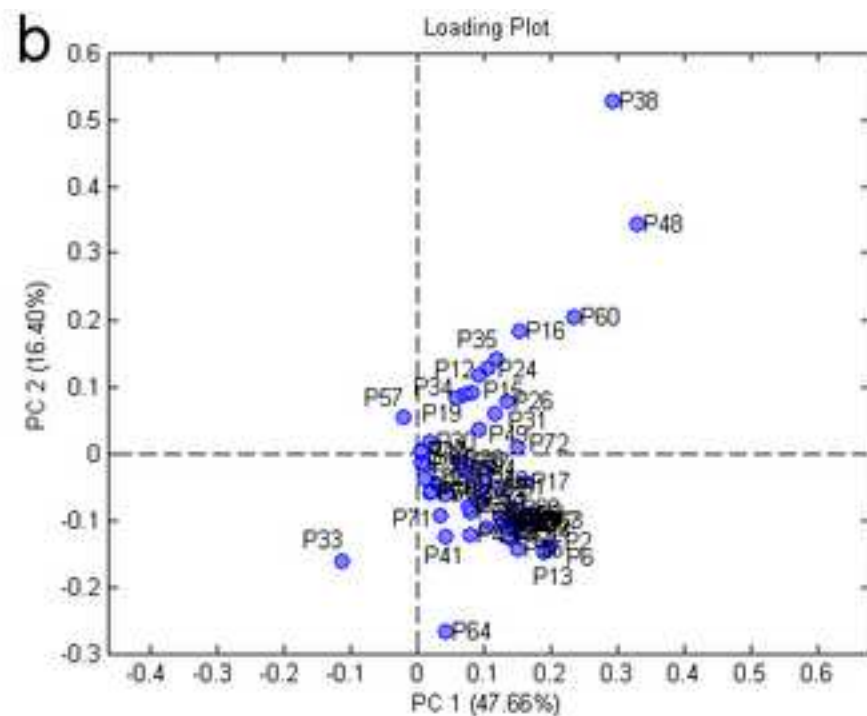
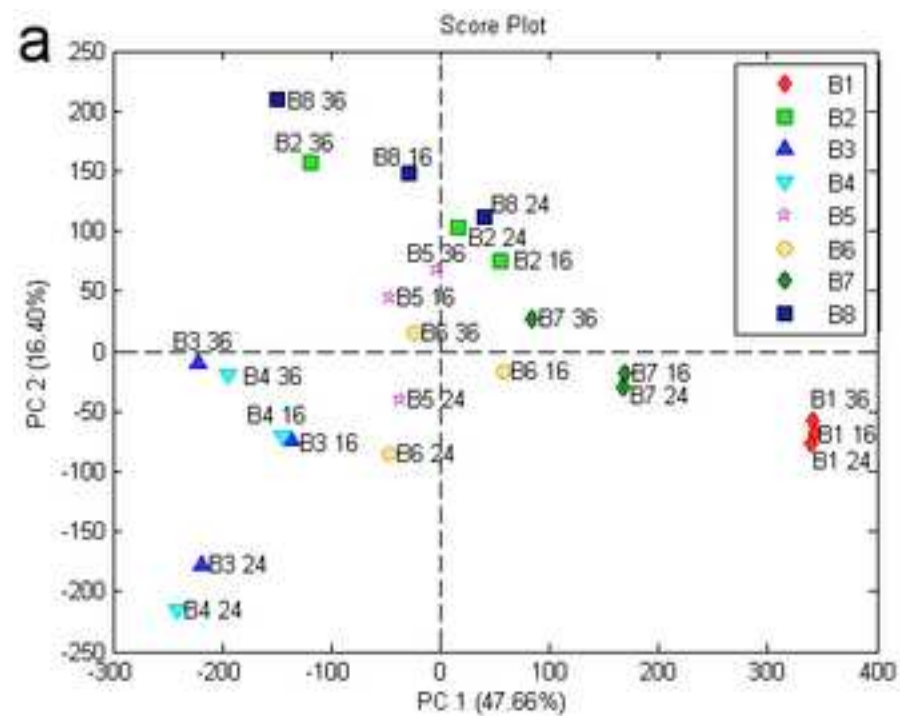


Figure 6  
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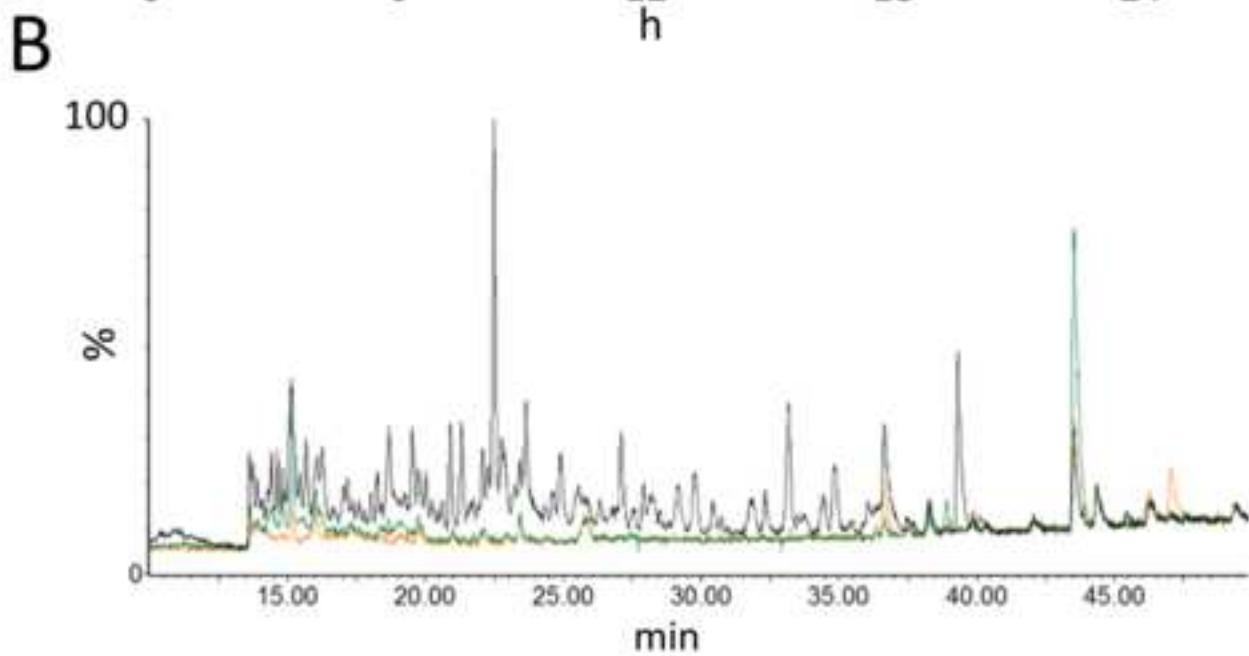
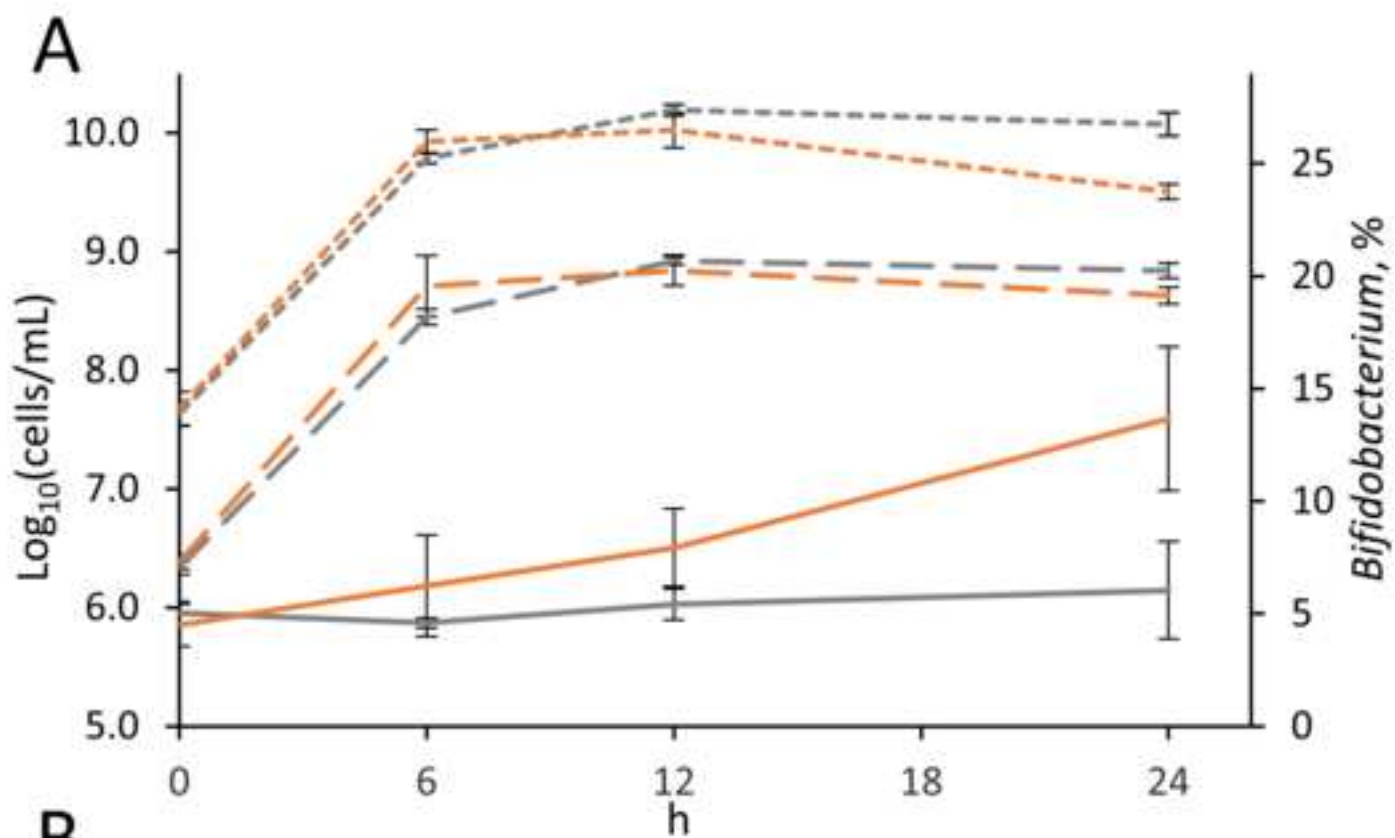


Table S1 List of the main peptides identified in water soluble extracts from PR at different ageing time. Characteristic fragments are listed using the classical fragmentation patterns of peptides. Underlined serines are bound to a phosphate group; -p indicates a phosphate group whose serine residue was not identified unambiguously. Numbers in brackets indicate the position of the fragmentation and the charge state of the fragment, when it is different from one. Retention time is referred to HPLC separation (total run time 110 min).

$r_T$ (min)	MW	SEQUENCE	a	b	c	x	y	z	PROTEIN
12.3	278	<b>EM</b>					150.0 (1)	262 (2)	$\gamma$ -Glu-Met
12.3	246	<b>EV</b>					118.1 (1)	230 (2)	$\gamma$ -Glu-Val
18.3	310	<b>EY</b>		129.8 (1)	146.9 (1)		182.1 (1)	165 (1)	$\gamma$ -Glu-Tyr
19.8	226	<b>EP</b>	83.8 (1); 181.1 (2)	209.1 (2)			115.9 (1)		pyro-Pro
20.8	242	<b>EL</b>	83.8 (1); 197.1 (2)	225.2 (2)	128.9 (1)		131.9 (1)		pyro-Leu
23.5	269	<b>NH</b>	224.1 (2)	252.1 (2)			156.0 (1)		
23.7	407	<b>EEM</b>					149.9 (1); 279.1 (2)	132.9 (1)	
24.1	230	<b>IV</b>	86 (1)				118.2 (1)		
24.6	375	<b>EEV</b>		130 (1); 358.5 (3)	147.8 (1)		247.1 (2); 118.0 (1)	230 (2)	
24.9	215	<b>Y</b>	170 (1)	198.1 (1)					
27.23	228	<b>EV</b>	83.7 (1); 183.1 (2)	211.1 (2)			118 (1)		pyro-Val
27.78	260	<b>EI</b>					131.9 (1)		$\gamma$ -Glu-ile
28.26	228	<b>LP</b>	183.1 (2)	211.2 (2)			229.2 (2); 115.7 (1)		
29.0	260	<b>EL</b>		129.9 (1)			132.0 (1)	244.1 (2)	$\gamma$ -Glu-leu
30.0	262	<b>IM</b>	86.2 (1); 216.9 (2)				149.9 (1)		
30.1	1155	<b>SSSEESITR</b>							
30.2	221	<b>N-lactosyl-Met</b>	176 (1)	204 (1)					
30.4	253	<b>N-lactosyl-Tyr</b>	208.1 (1)	236.1 (2)					
31.3	294	<b>EF</b>		129.9 (1)			166 (1)	278 (2)	$\gamma$ -Glu-Phe
32.4	189	<b>N-lactosyl-Val</b>	144 (1)	172 (1)					
32.7	389	<b>EEI</b>		130 (1)			261.2 (2); 132.1 (1)	244.3 (2)	
33.9	1348	<b>LSSSEESITR</b>	86 (1)				605.2 (5); 476.3 (4); 276.1 (2)		$\beta$ -casein
34.2	571	<b>EIVPN</b>					230.1 (2); 329.1 (3)		$\alpha_{S1}$ -casein
34.6	242	<b>EI</b>	83.7 (1); 197.1 (2)	225.2 (2)	128.8 (1)		131.8 (1)		pyro-Ile
35.5	333	<b>EW</b>		129.8 (1)			205.3 (1)	317.3 (2); 188.1 (1)	
36.1	1034	<b>SSEEIVPN</b>	777.2 (6)	706.1 (5); 805.1 (6)			571 (5); 329.2 (3); 230 (2); 133.7 (1)		$\alpha_{S1}$ -casein
36.4	242	<b>EL</b>	83.7 (1); 197.1 (2)	225.2 (2)	128.8 (1)		131.8 (1)		pyro-Leu
36.6	954	<b>SSEEIVPN</b>		725.2 (6)			329 (3); 230.6 (2)		$\alpha_{S1}$ -casein
36.8	244	<b>IL</b>	86.0 (1); 199.0 (2)	227.1 (2)	131.6 (1)		132 (1)		
37.8	278	<b>FI</b>	119.7 (1); 233.2 (2)	261.1 (2)			132 (1); 279.2 (2)		
38.9	278	<b>IF</b>	85.8 (1)				166 (1)		
39.1	1702	<b>PSGAWYYVPLGTQYT</b>		711.9 (13, 2+)			760.9 (13, 2+); 536 (14, 3+)		$\alpha_{S2}$ -casein
40.1	203	<b>N-lactosyl-Ile/Leu</b>							
40.2	1999	<b>ESLSSSEESITRINK</b>	101.9 (1)	628.7 (9, 2+)			530.8 (4); 416.9 (7, 357 (3); 563.4 (13, 2+); 796.4 (12, 2+); 3+)		$\beta$ -casein
							530.8 (12, 3+)		

40.4	1870	QFLPYPIYAKPAAVRS		129.1 (1)		262.4 (2)		$\alpha_{S1}$ -casein
40.5	278	FI/L		120.0 (1); 233.4 (2)		131.8 (1)		
40.6	2051	KHYQKALNEINQFYQK				585.4 (4)		$\alpha_{S2}$ -casein
41.0	278	LF		86 (1)		166 (1)		
42.6	237	N-lactosyl-Phe		192.1 (1)	220.1 (1)	238.1 (1)		
43.5	2264	PQLEIVPNSAEERLHSMKEG		912.9 (16, 2+)		723.3 (19, 3+)	524 (14, 3+)	$\alpha_{S1}$ -casein
45	1938	TQTPVVVPPFLQPEVMGV			230.2 (2)	571.8 (10, 2+)	855.6 (16, 2+)	$\beta$ -casein
45.9	2877	SLSQSKVLPVPQKAVPYPQRDMPIQA		201.2 (6, 3+)	201.2 (2); 100.7 (2, 218.3 (2); 520.1 (10, 2+); 520.1 (10, 2+); (5); 428.6 (12, 3+)	428.6 (7, 2+)	218.3 (2); 428.6 (4) 201.2 (2); 100.7 (2, 2+); 520.1 (9, 2+); 887.8 (24, 3+)	
46.4	2247	VLPVPQKAVPYPQRDMPIQA		71.8 (1)	213.4 (2)	528.5 (9); 659.1 (11, 2+); 1018.2 (18, 2+)		$\beta$ -casein
47.1	3579	EELNVPGEIVESLSSSEESITRINKKIEK		955.1 (9)	486.8 (4); 244.1 (4, 146.9 (1) 2+); 829.8 (14, 2+); 404.8 (11, 3+)	754 (13, 2+)	146.9 (1); 486.8 (12, 3+); 791.1 (18)	$\beta$ -casein
49.2	2763	RPKHPIKHQGLPQEVLENLLRF		226.2 (2)	647.5 (11, 2+); 873.8 (15, 2+); 582.8 (15, 3+); 550 (14, 3+)	932.4 (7)	662.5 (5); 1018.6 (17, 2+); 183.2 (4, 3+)	$\alpha_{S1}$ -casein
49.4	3451	DAYPSGAWYYVPLGTQYTDAPSFSDIPNIGS			322.2 (3)	650.8 (17)	905.6 (17)	$\alpha_{S2}$ -casein
49.9	1812	YQGPIVLNPWDQVKR		135.9 (1); 264.2 (2)	292.2 (2); 349.1 (3); 599.4 (15, 3+)		928.6 (7); 465 (7, 2+); 733.1 (12, 3+); 599.4 (15, 3+)	
50.5	5948	QEVLENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQMEAES		946.9 (16, 2+); 1336.6 (35)	128.9 (1); 679.3 (11, 2+)	595 (15, 3+)	656.2 (11, 2+)	$\alpha_{S1}$ -casein
51.5	391	FLL		119.9 (1); 233.2 (2)	261.1 (2)		132.2 (1); 245.1 (2) 375.1 (3)	
52.1	1707	FVAPFPEVFGKEK			247 (2); 318.1 (3)		731.4 (13, 2+); 695.7 (12); 488 (13, 3+)	$\alpha_{S1}$ -casein
52.4	7182	KEAMAPKHKEMPPFKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQ		451.3 (12, 3+); 500.6 (13, 3+)	129.2 (1); 812.4 (14, 2+)	135.8 (2, 2+)	451.3 (12, 3+)	$\beta$ -casein
52.5	3193	KEKVNELSKDIGSESTEDQAMEDIKQ			129.1 (1); 129.1 (2, 2+); 129.1 (3, 3+); 810.4 (20, 3+)	911.8 (22, 3+)	129.1 (2, 2+)	$\alpha_{S1}$ -casein
52.7	7054	EAMAPKHKEMPPFKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQ		810.5 (21, 3+)		259.8 (5); 633.4 (16, 3+)	917.0 (8); 677.2 (12, 2+); 451.4 (12, 3+)	$\beta$ -casein
54.2	5486	STEDQAMEDIKQMEAESISSSEEIVPNSVEQKH IQKEDVP SERYL				739.9 (18, 3+)	739.9 (12, 2+); 493.8 (12, 3+)	
55.1	1237	FVAPFPEVFGK			247.1 (2); 318.1 (3)		920.3 (8); 991.4 (9); 496.1 (9, 2+); 460.7 (8, 2+)	$\alpha_{S1}$ -casein
55.2	4237	HIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPN		110 (1); 411.9 (2+,7)	251.2 (2); 426 (2+,7); 251.2 (6, 3+)	86 (2, 3+)	133.1 (1); 230.2 (2); 110 (3, 3+)	$\alpha_{S1}$ -casein
56.4	3858	KEDVPSERYLGYLEQLLRLKKYKVPQLEIVPN		230.2 (2)	129.1 (1); 129.1 (2, 2+); 908.9 (22, 3+)	1162.6 (10)	133.1 (1); 230.2 (2); 329.3 (3);	

							909.5 (8); 1162.6 (29, 3+)		
<b>57.4</b>	12262	<b>KHPIKHQGLPQEVLNENLLRFFVAPFPEVFGK EKVNELSKDIGSESTEDQAMEDIKQMEAESISS SEEIVPNSVEQKHIQKEDVPSERYLGYLEQLLR LKKYK</b>		129.2 (1)			1343.9 (33, 3+)	$\alpha_{S1}$ -casein	
<b>58.0</b>	3602	<b>KEDVPSERYLGYLEQLLRLKKYKVPQLEIVPN</b>	187.1 (2); 1082 (27, 3+)	230.1 (2)	326 (8, 3+)	565.3 (14, 3+)		1082 (18, 2+)	
<b>60.1</b>	1881	<b>YQQPVLGPVRGPFPIIV</b>	136.1 (1); 1264.9 (12)	292.2 (2); 421.2 (3); 616.1 (5)	181.1 (1)		441.4 (4); 1094.7 (10); 1151.7 (11); 1264.9 (12); 1461.8 (14); 731.3 (14, 2+)		
<b>60.1</b>	1882	<b>LEELNVPGEIVESLSSS-p</b>	85.8 (1)	1094.7 (10)	616.1 (5)		181.1 (3, 2+)		
<b>61.8</b>	8715	<b>MGVSKVKEAMAPKHKEMPFPKYVPEPFTESQ SLTLTDVENLHLPLPLQSWMHQPHQLPPTV MFPPQSVLSLSQSK</b>	1106.7 (11); 453.1 (9, 2+); 1100.3 (20, 2+); 620.4 (17, 3+); 774.7 (21, 3+)	365.3 (7, 2+); 1658.6 (29, 2+); 453.1 (13, 3+); 1106.7 (29, 3+); 1801 (48, 3+)	620.4 (6); 756.1 (14, 2+)		762.2 (7); 774.7 (14, 2+); 1391.2 (25, 2+); 762.2 (21, 3+); 1106.7 (29, 3+); 1142.5 (30, 3+)	1649.8 (29, 2+); 756.1 (21, 3+); 1513.6 (40, 3+)	$\beta$ -casein
<b>68.3</b>	4023	<b>SLVYFPFGPIPNLQNIPLTQTPVVPPFLQP EVM</b>	227.2 (6, 3+)	707.4 (6); 1071.7 (10); 1936 (18); 968.3 (18); 1483.9 (28, 2+)	326.9 (2, 3+)	1058.6 (19, 2+)	475.3 (4); 1057.4 (9)	908.7 (27, 3+)	

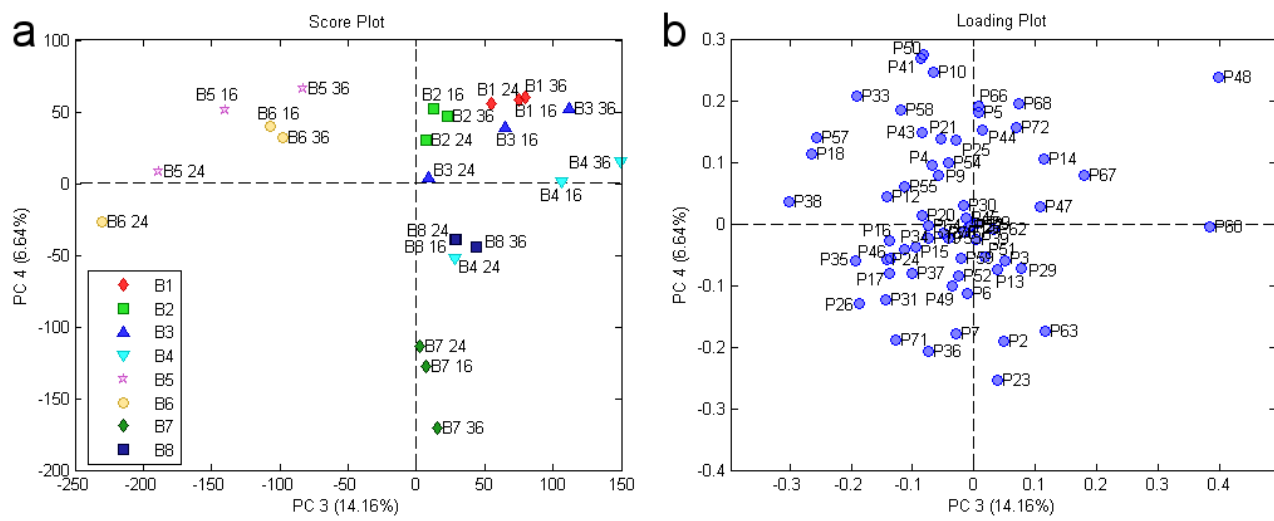
Table S2. List of the main peptides identified in digested samples of PR at different ageing time. Characteristic fragments are listed using the classical fragmentation patterns of peptides. Underlined serines are bound to a phosphate group; -p indicates a phosphate group whose serine residue was not identified unambiguously. Numbers in brackets indicate the position of the fragmentation and the charge state of the fragment, when it is different from one. Retention time is referred to HPLC separation (total run time 110 min).

#	r <sub>T</sub> (min)	MW	SEQUENCE	Precursor Ion	a	b	c	P	y	z	PROTEIN
P1	9.9	614	<b>LHSMK</b>	308	85.8 (1)	251.7 (2)			147.1 (1); 365.3 (3); 502.2 (5); 251.7 (4, 2+)		β-casein
P2	10.23	909	<b>EGIHAQQK</b>	455		130.1 (1); 187.2 (2)			474.3 (4); 611.4 (5)		α <sub>s1</sub> -casein
P3	17.8	715	<b>n. d.</b>								
P4	21.7	230	<b>IV</b>	231	86.1 (1); 185.2 (2)						
P5	23	548	<b>YAKPA</b>	549		363.1 (3); 531.3 (5)			315.2 (3)		κ-casein
P6	25.7	781	<b>EGIHAQQ</b>	782		437.3 (4); 508.3 (5); 636.5 (6); 764.5 (7)			782.4 (7); 653.4 (6); 483.3 (4); 275.0 (2); 147.1 (1)		α <sub>s1</sub> -casein
P7	27.6	830	<b>EDVPSE</b>	831					175.2 (1); 587.3 (5)	471.2 (4)	α <sub>s1</sub> -casein
P8	27.6	260	<b>EL</b>	261		130.0 (1)			132.8 (1)	244.1 (2)	
P9	27.7	230	<b>VI</b>	231	71.9 (1); 185.2 (2)				132 (1)		
P10	27.8	351	<b>YIG</b>	352		277.2 (2)			189.1 (2)		
P11	27.8	526	<b>EGIHA</b>	525		187.0 (2); 437.2 (4); 508.3 (5)			227.2 (2); 340.4 (3)		α <sub>s1</sub> -casein
P12	27.9	388	<b>QEL</b>	389	101.9 (1)	129.0 (1); 258.2 (2)			132.0 (1)		α <sub>s1</sub> -casein
P13	28.4	1336	<b>n. d.</b>								
P14	29.7	1180	<b>HIQKEDVPSE</b>	591	109.9 (1); 223.2 (2); 919.6 (8)	751.2 (6); 850.5 (7); 474.3 (8, 2+)			235.3 (2); 332.2 (3)	109.9 (2, 2+); 583.1 (10, 2+)	κ-casein
P15	30	663	<b>TESQSL</b>	664	73.6 (1); 203.2 (2)	231.1 (2); 446.2 (4); 533.2 (5); 646.3 (6)			347.2 (3); 434.3 (4);		β-casein
P16	30.55	706	<b>AMEDIK</b>	705	175.1 (2); 532.2 (5)	203.2 (2); 332.1 (3); 447.2 (4); 560.3 (5);			147.0 (1); 260.3 (2); 375.2 (3); 504.3 (4);		α <sub>s1</sub> -casein
P17	30.7	679	<b>DAYPSGA</b>	678		115.9 (1); 187.0 (2); 350.1 (3)			331.3 (4); 234.2 (3)		α <sub>s1</sub> -casein
P18	30.8	276	<b>AW</b>	275	230.2 (2)	258.2 (2)			205.2 (1)	188.2 (1)	
P19	31.1	633	<b>YKVPQ</b>	634	292.1 (2);				343.3 (3); 244.2 (2); 147.2 (1)		α <sub>s1</sub> -casein
P20	31.4	294	<b>YI / IY / YL / LY</b>	295	136.0 (1); 249.2 (2)				132 (1)	278.2 (2)	
P21	32.2	330	<b>PYPQR</b>	659		261.1 (2)			563.3 (4); 400.2 (3); 175.2 (1)		β-casein
P22	32.4	420	<b>QSW</b>	419	100.8 (1); 188.1 (2)	129.9 (1); 216.2 (2); 402.2 (3)	146.2 (1)		205.2 (1); 292.2 (2)	188.2 (1)	β-casein
P23	32.8	507	<b>HKEMPFK</b>	1012	110.1 (1); 371.7 (6, 2+)		155.2 (1)		147 (1); 244.3 (2); 391.3 (3); 488.2 (4); 876.3 (7); 244.3 (4, 2+)		β-casein
P24	34.3	676	<b>TLTDVE</b>	677	187.2 (2); 502.2 (5)	215.3 (2); 316.2 (3); 231.3 (4); 530.3 (5); 659.3 (6)			463.3 (4); 362.2 (3); 247.2 (2); 148.0 (1)		β-casein
P25	34.5	779	<b>VLPVPQK</b>	780					372.3 (3); 568.4 (5)		β-casein
P26	34.7	675	<b>AVPYPQ</b>	674		431.2 (4);			244.1 (2); 504.2 (4)		β-casein
P27	36.7	512	<b>IHPF</b>	513					166.0 (1); 263.0 (2)		β-casein
P28	37	501	<b>LEQL</b>	502		243.1 (2); 371.1 (3); 484.3 (4)			260.2 (2)	243.1 (2)	α <sub>s2</sub> -casein

P29	37.7	812	<b>NAVPIPTPT</b>	811		285.1 (3); 495.3 (5); 596.4 (6);	627.5 (6); 528.3 (5); 318.2 (3); 217.1 (2);		$\alpha_{s2}$ -casein
P30	38.7	558	<b>ALNEI</b>	559	157.2 (2)	185.3 (2); 299.2 (3); 428.4 (4)	375.2 (3); 261.2 (2);	542.3 (5); 358.2 (3); 244.2 (2)	$\alpha_{s2}$ -casein
P31	39.1	674	<b>DMPIQA</b>	673		247.1 (2); 344.2 (3); 457.2 (4); 585.3 (5)	428.3 (4); 218.2 (2);	411.2 (4);	$\beta$ -casein
P32	39.4	651	<b>VLPVPQ</b>	652		310.2 (3); 409.3 (4);	244.1 (2)		$\beta$ -casein
P33	40.7	1512	<b>MHQPHQPLPPTVM</b>	757		269.2 (2); 397.2 (3); 759.4 (6); 969.4 (8); 1066.3 (9); 1263.9 (11)	544.3 (5); 447.4 (4); 150.0 (1)		$\beta$ -casein
P34	41	562	<b>TTMPL</b>	561			360.2 (3); 229.3 (2)		$\alpha_{s1}$ -casein
P35	42.7	587	<b>PVEPF</b>	588		326.2 (3);	1661. (1); 263.3 (2); 392.2 (3);		$\beta$ -casein
P36	43.7	754	<b>SDIPNPI</b>	755		527.3 (5)	553.5 (5); 440.3 (4); 229.2 (2)	738.6 (7); 212.1 (2)	$\alpha_{s1}$ -casein
P37	43.7	645	<b>EPMIGV</b>	644		358.1 (3); 471.2 (4); 528.2 (5); 627.3 (5)	419.2 (4); 288.2 (3); 175.2 (2);		$\alpha_{s1}$ -casein
P38	44.9	504	<b>YIPI</b>	505	136.1 (1); 249.1 (2)		131.9 (1); 229.4 (2);		$\kappa$ -casein
P39	45.2	729	<b>Q/KNDEPK/Q</b>	730	459.2 (4)	129.1 (1)	147.1 (1); 244.3 (2); 488.4 (4)		
P40	45.6	685	<b>RGPFPI</b>	686		157.1 (1);	229.2 (2)	669.2 (6)	$\beta$ -casein
P41	45.7	1139	<b>IASGEPTSTPT</b>	571		635.5 (5)	217.1 (2); 317.8 (3);		$\alpha_{s1}$ -casein
P42	46	721	<b>GYLEQL</b>	722	563.4 (5)	221.2 (2); 334.1 (3); 463.3 (4); 591.3 (5); 704.3 (5)	502.3 (4); 389.4 (3); 260.3 (2);	243.2 (2)	$\alpha_{s1}$ -casein
P43	46.7	750	<b>YPVEPF</b>	751	136.1 (1); 233.2 (2); 461.3 (4)	261.2 (2); 360.2 (3); 489.2 (4)	166.1 (1); 263.2 (2); 392.2 (3); 588.2 (5)		$\beta$ -casein
P44	47.6	1462	<b>EEYSIGSSSEES</b>	732			698.6 (5)		$\alpha_{s2}$ -casein
P45	48.1	653	<b>YYVPL</b>	654		426.3 (3)	229.2(2); 328.3 (3)		$\alpha_{s1}$ -casein
P46	48.6	757	<b>VAPFPEV</b>	758	387.3 (4)	268.2 (3); 415.2 (5);	344.2 (3); 491.3 (4); 588.3 (5);		$\alpha_{s1}$ -casein
P47	48.9	820	<b>VLSRYPS</b>	821	272.3 (3)		609.2 (5); 522.2 (4)		$\alpha_{s1}$ -casein
P48	49	530	<b>GPFPI</b>	520		155 (2); 302.2 (3)	376.3 (3); 229.3 (2)		$\beta$ -casein
P49	50	688	<b>LHLPLP</b>	689		251.2 (2); 461.3 (4);	229.2 (2); 439.3 (4);		$\beta$ -casein
P50	51	1100	<b>VYFPFGPIP</b>	1099	730.4 (7)	871.5 (8)	230.3 (2); 440.2 (4); 594.4 (6); 838.5 (8)	1083.6 (10)	$\beta$ -casein
P51	52.3	1372	<b>TDAPSFSDIPNPI</b>	1373		821.7 (8); 934.6 (9)	229.3 (2); 440.3 (4); 553.5 (5); 755.7 (7); 1087.6 (10); 1157.9 (11)	212.2 (2); 1356.7 (13)	$\alpha_{s1}$ -casein
P52	52.4	1267	<b>INNQLFPYPY</b>	1268		730.5 (6); 990.6 (8)	279.3 (2); 539.3 (4); 652.4 (5)		$\kappa$ -casein
P53	52.8	829	<b>YQGPIVLNPWDQVK</b>	1656	136.1 (1)	292.2 (2); 349.1 (3); 658.3 (6)	772.5 (6); 1000.6 (8); 1309.9 (11); 1366.3 (12);		$\alpha_{s2}$ -casein
P54	53.7	2429	<b>HIQKEDVPSERYLGYLEQLL</b>	1216		741.5 (12); 827.1 (14)		1017.9 (17)	$\alpha_{s1}$ -casein
P55	54.3	1479	<b>EMPFPKYPVEPF</b>	740		1218.8 (10)	166.2 (1); 263.3 (2); 976.7 (8); 611 (10, 2+)	958.8 (8)	$\beta$ -casein
P56	54.4	888	<b>VYFPFGPI</b>	889			383.3 (4); 627.3 (7)		$\beta$ -casein
P57	54.6	904	<b>FVAPFPEV</b>	905		415.3 (4); 562.3 (5); 659.4 (6)	344.1 (3); 491.3 (4); 588.3 (5); 659.4 (6)		$\alpha_{s1}$ -casein
P58	54.8	1299	<b>SLVYFPFGPIP</b>	1300		804.3 (7); 1071.6 (10)	594.4 (6); 838.5 (8); 1001.6 (9)		$\beta$ -casein
P59	55.1	1236	<b>FVAPFPEVFGK</b>	619	119.9 (1); 219.3 (2); 316.1 (6, 2+)	247.2 (2); 318.2 (3)	676.5 (6); 823.4 (7); 920.5 (8); 991.5 (9); 412.3 (7, 2+); 460.8 (8, 2+); 496.4 (9, 2+)		$\alpha_{s1}$ -casein



P60	55.1	1248	<b>PVLGPVVRGPFPI</b>	625	169.2 (2); 339.3 (4); 85.7 (2, 2+); 497.2 (10, 2+)	197.2 (2); 310.3 (3); 719.5 (7); 1020.7 (10); 559.3 (11, 2+)		229.3 (2); 882.5 (8); 939.5 (9); 1052.7 (10); 441.7 (8, 2+); 527.2 (10, 2+)		$\beta$ -casein
P61	55.2	753	<b>QEPVLGPVVRGPFPI</b>	1505		624.7 (6); 744.4 (14, 2+)		229.3 (2); 882.6 (8); 939.7 (9); 1053.9 (10); 1152.5 (11); 1250.1 (12); 624.8 (12, 2+)	744.4 (14, 2+)	$\beta$ -casein
P62	55.7	643	<b>GPFPII</b>	642		155.1 (2); 302.3 (3); 399.4 (4); 512.3 (6)		489.4 (4); 342.3 (3); 245.1 (2);		$\beta$ -casein
P63	56.6	1220	<b>PVVVPPFLQPE</b>	1221		296.5 (3); 395.3 (4); 492.4 (5); 736.6 (7); 849.7 (8); 977.7 (9)		827.6 (7)		$\beta$ -casein
P64	57.5	741	<b>GPFPIIV</b>	742		625.4 (6); 512.3 (5);		441.3 (4);		$\beta$ -casein
P65	60.1	1882	<b>LEELNVPGEIVESLSSS-p</b>		85.8 (1)	1094.7 (10)	616.1 (5)	181.1 (3, 2+)		
P66	60.6	1360	<b>TDVENLHLPLPL</b>	681		217.2 (2); 922.5 (8); 1132.7 (10)		229.3 (2)		$\beta$ -casein
P67	61.3	1258	<b>DVENLHLPLPL</b>	630	187.2 (2)	821.5 (7); 1031.7 (9)		229.3 (2)		$\beta$ -casein
P68	61.7	788	<b>LLDAQSAPLRVYVE</b>	1573	85.8 (1)			1347 (12)		$\beta$ -lactoglobulin
P69	61.8	2511	<b>SLPQNIPPLTQTPVVPPFLQPE</b>	1257	926.6 (18, 2+)	653.6 (6); 1585.7 (15); 1685 (16); 1135.5 (21, 2+); 1248.4 (23, 2+)	1248.4 (13)	148.2 (1); 245.3 (2); 373.4 (3); 486 (4); 827.6 (7); 926.5 (8); 1322.6 (12); 1451 (13)	653.6 (12, 2+)	$\beta$ -casein
P70	63.4	2610	<b>SLPQNIPPLTQTPVVPPFLQPEV</b>	1306		653.5 (6); 1090.1 (11); 1587.1 (15); 1686.3 (16)	766.7 (7)	344.3 (3); 472.5 (4); 926.7 (8); 1025.8 (9); 1321.1 (12); 1958.3 (18)		$\beta$ -casein
P71	63.5	1700	<b>n. d.</b>							
P72	63.9	1774	<b>n. d.</b>							



**Figure S1.** PCA model calculated on *Bifidobacterium* strains: PC3-PC4 score plot (a) and loading plot (b).