This is the peer reviewd version of the followng article:

Characterization of the peptide fraction from digested Parmigiano Reggiano cheese and its effect on growth of lactobacilli and bifidobacteria / Bottari, Benedetta; Quartieri, Andrea; Prandi, Barbara; Raimondi, Stefano; Leonardi, Alan; Rossi, Maddalena; Ulrici, Alessandro; Gatti, Monica; Sforza, Stefano; Nocetti, Marco; Amaretti, Alberto. - In: INTERNATIONAL JOURNAL OF FOOD MICROBIOLOGY. - ISSN 0168-1605. - 255:(2017), pp. 32-41. [10.1016/j.ijfoodmicro.2017.05.015]

Terms of use:

The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

01/05/2024 04:03

1 Characterization of the peptide fraction from digested Parmigiano Reggiano cheese and its effect on

2 growth of lactobacilli and bifidobacteria

- 3 Benedetta Bottari^b, Andrea Quartieri^a; Barbara Prandi^b; Stefano Raimondi^a; Alan Leonardi^a; Maddalena
- 4 Rossi^{a,c}, Alessandro Ulrici^{a,c}; Monica Gatti^{b,d}; Stefano Sforza^b; Marco Nocetti^e; Alberto Amaretti^{a,c}
- ^a Department of Life Sciences, University of Modena and Reggio Emilia, Italy
- 6 ^b Department of Food Science, University of Parma, Italy
- ^c Biogest-Siteia, Centro per il Miglioramento e la Valorizzazione delle Risorse Biologiche Agro-Alimentari,
- 8 University of Modena and Reggio Emilia, Italy
- 9 ^d Multidisciplinary Interdepartmental Dairy Center MILC, University of Parma, Parma, Italy
- 10 ^e Servizio Tecnico Consorzio del Formaggio Parmigiano Reggiano, Reggio Emilia, Italy

11

- 12 Correspondence: Alberto Amaretti, PhD, Dept. Life Sciences, University of Modena and Reggio Emilia,
- 13 Via G. Campi 103, 41125 Modena, Italy, alberto.amaretti@unimore.it

15 Abstract

16 Parmigiano Reggiano (PR) is a raw-milk, hard cooked, long-ripened cheese of high quality and nutritional value. Long ripening times allow for extensive proteolysis of milk proteins to yield a number of peptides, 17 some of which have potential healthy bioactive properties. This study aimed to: i) determine the peptide 18 profile of PR cheese subjected to simulated gastrointestinal transit; ii) evaluate in vitro whether the peptides 19 could support growth of beneficial microbial groups of the gut microbiota. PR samples were subjected to in 20 vitro digestion, simulating oral, gastric, and duodenal transit. Liquid chromatography coupled with tandem 21 mass spectrometry revealed that digestion caused the disappearance of the serum proteins and most of the 22 23 original peptides, while 71 new peptides were found, all ranging from 2 to 24 residues. The digests were given as sole nitrogen source to pure cultures of *Bifidobacterium* (27 strains) and *Lactobacillus* (30 strains), 24 and to bioreactor batch cultures of human gut microbiota. Most of bifidobacteria and lactobacilli grew more 25 abundantly on PR digests than on the control peptone, and exhibited strain- or species- specific peptide 26 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 homogenously 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 abundance of residues with peculiar properties (hydrophobicity, polarity, charge) and likely depend on 33 34 specific proteases and/or peptide transporters preferentially recognizing specific sequence motifs. The cultures of human colonic microbiota confirmed that PR digest promoted the growth of commensal 35 bifidobacteria. This study demonstrated that peptides derived from simulated gastrointestinal digestion of PR 36 supported the growth of most lactobacilli and bifidobacteria. 37

38

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 nutritional value, produced in a restricted area in northern Italy. PR is produced from bovine milk with rennet 54 and natural whey starter and a Protected Designation of Origin, in compliance with the European norm 55 currently in force (European Commission, 2009, 2011). The curd is heated at 55°C to select thermophilic 56 57 bacterial strains and it is ripened at least for 12 months, even if much longer ageing times are usually adopted. In mature PR, the moisture is 28 to 35%. The dry weight is mostly composed of proteins and lipids 58 fractions, the ratio of fat to protein being around 0.94, depending on milk characteristics. Ripened PR is 59 60 lactose- and galactose-free and rich in free organic acids, mostly derived from bacterial fermentations, such as lactic acid (1.5 g per 100 g of PR), citric (50 mg), acetic (100 mg), propionic (0.5 mg), and butyric (120 61 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 aminoacyl derivatives also accumulate (Sforza et al., 2009, 2012). Casein breakdown contributes to 68 improved digestibility, reduced allergenicity, and flavor development (Alessandri et al., 2012; Sforza et al., 69 2012). The potential biological activity of PR peptides has recently attracted particular interest (Korhonen et 70 al., 2009; Tidona et al., 2009). In vitro studies revealed calcium binding properties (Kim et al., 2004; Pinto et 71 al., 2012) and antioxidant (Bottesini et al. 2013; Gupta et al., 2009), antihypertensive (Bernabucci et al., 72 73 2014), antimicrobial activities (Benkerroum, 2010; Rizzello et al., 2005)... The nutritional value of PR, coupled with the potential beneficial properties of bioactive peptides 74

75 that could impact health, suggested its use as functional food in a dietary therapy for subjects with

real resulting from alimentary intolerance, post-therapeutic antibiotic-associated

dismicrobism, or post-infective conditions (Olivi et al., 1979; Pancaldi et al., 2008). In particular, a 77 78 homemade food based on PR cheese was developed and successfully utilized to feed infants suffering from different forms of intestinal problems, with a rapid clinical improvement and normalization of the gut. The 79 high digestibility and the high amounts of short chain fatty acids, amino acids, and oligopeptides easily 80 absorbed in the bowel likely support this beneficial effect. Furthermore, the hypoallergenicity, the absence of 81 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). Previous studies demonstrated that the hydrolysates of proteins from different origins, and particularly from 88 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). The health effects of PR bioactive peptides on intestinal bacteria require that they resist 95 gastrointestinal digestion and, at least partially, reach the colon. The present study aimed to characterize the 96 peptides derived from simulated gastrointestinal digestion of PR cheese, utilizing an in vitro digestion model 97 that recently achieved an international consensus (Minekus et al., 2014). The resulting peptide mixtures were 98 characterized by liquid chromatography coupled with mass spectrometry. In order to verify whether peptides 99 specifically promoting the growth of beneficial bacteria and potential probiotics could be identified, the 100 101 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*

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

114

115 *2.1 Simulated gastrointestinal digestion of PR samples*

All the chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) unless otherwise 116 stated. The cheese was finely grounded and digested following the procedure, consisting in three main steps: 117 118 salivary phase, gastric phase, and intestinal phase (Minekus et al., 2014). A sample of 25 g cheese was added to 17.5 mL of salivary buffer (15.1 mM KCl, 3.7 mM KH₂PO₄, 13.6 mM NaHCO₃, 0.15 mM MgCl₂, and 119 0.06 mM (NH₄)₂CO₃), 2.5 mL of 1500 U/mL amylase, 125 µL of 300 mM CaCl₂, and 4.875 mL of distilled 120 water (ratio cheese:digestive fluid of 1:1, w:v). The sample was vortexed and incubated for 2 min at 37°C on 121 a reciprocating shaker. Then, 37.5 mL of gastric buffer (6.9 mM KCl, 0.9 mM KH₂PO₄, 25 mM NaHCO₃, 122 47.2 mM NaCl, 0.1 mM MgCl₂, and 0.5 mM (NH₄)₂CO₃), 8 mL of 25000 U/mL pepsin, 25 µL of 300 mM 123 CaCl₂, 1 mL of 1 M HCl and 3.475 mL of water were added (final ratio cheese:digestive fluids 1:3, w:v). 124 125 The pH was adjusted to 3 with 1 M HCl. The mixture was vortexed and incubated for 2 h at 37°C on a reciprocating shaker. Finally, 55 mL of intestinal buffer (6.8 mM KCl, 0.8 mM KH₂PO₄, 85 mM NaHCO₃, 126

127	38.4 mM NaCl, 0.33 mM MgCl ₂), 25 mL of 800 U/mL pancreatin, 12.5 mL of 75 mg/mL bile solution, 200
128	μL of 300 mM CaCl_2, 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 \times g to remove insoluble proteins and
133	undigested components (Minekus et al., 2014). The supernatant was filtered through 0.45 μ m membranes.
134	For chromatographic analysis, 196 μ L of sample was supplemented with 4 μ 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 μ m
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
	2000 III 2), eupinitity voltage 212 k + , cone voltage 20 + , source temperature 100 °C, desorvation temperature

Peptides were manually identified as follows. Molecular ions were recorded from the Full Scan 152 153 chromatogram, then they were fragmented in Daughter Scan modality using the triple quadrupole equipment. The MW of each peptide was launched in FindPept tool (http://web.expasy.org/findpept last accessed on 154 155 September 2016) against the main milk proteins (α -casein, β -casein, κ -casein, α -lactalbumin and β lactoglobulin), to obtain a list of peptide sequences with a compatible MW. The theoretical fragmentation 156 pattern of these sequences was determined 157 (http://db.systemsbiology.net/proteomicsToolkit/FragIonServlet.html last accessed on September 2016) and 158 159 compared with the MS/MS spectra. The peptide sequences were assigned on the basis of the matching 160 fragments. 161 2.3 UPLC/ESI-MS analysis. 162 Absolute quantification of all the peptides identified was not possible due to the unavailability of all 163 164 the peptide standards, and a direct comparison of the peptide areas would have been inaccurate due to differences in ionization efficiency. Once the sequences were assigned, semi-quantitative analysis was 165 performed using Phe-Phe as internal standard. The ratio between the chromatographic peak of the peptide 166 and that of Phe-Phe did not yield absolute peptide concentration but allowed the comparison of the same 167 168 peptide in different samples. For the semi-quantitative analysis, UPLC (Ultra Performance Liquid Chromatography) was chosen instead of HPLC, given the higher chromatographic resolution. Samples were 169 separated by a reverse phase column (Acquity UPLC BEH 300 C18, 1.7 μ m, 2.1 \times 150 mm equipped with a 170 Acquity UPLC BEH C18 VanGuard Pre-column, 300Å, 1.7 μm, 2.1 × 5 mm, Waters) in an UPLC system 171 coupled with ESI and MS (UPLC Acquity with a single quadrupole detector SQD, Waters). In order to 172

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;

	9
201	Growth of bifidobacteria was determined in triplicate by measuring the turbidity at 600 nm (OD_{600})
200	peptide consumption.
199	based media were incubated 24 h at 37°C, and passaged at least three times before evaluation of growth and
198	H_2O , 0.05 g/L; cysteine hydrochloride, 0.5 g/l; pH was corrected to 6.5. Bacteria inoculated (5% v/v) in BM
197	g/L; polysorbate 80, 1 g/L; sodium acetate trihydrate, 5 g/L; K_2 HPO ₄ , 2 g/l; MgSO ₄ · 7H ₂ O, 0.1 g/l; MnSO ₄ ·
196	g/L; ammonium citrate, 2 g/L; yeast nitrogen base w/o amino acids and ammonium sulfate (BD Difco), 1.7
195	positive control. The medium contained lactose, 10 g/L; PR hydrolysates or Bacto peptone (BD Difco), 5
194	PR16, PR24, PR36 digests or Bacto peptone (BD Difco) were utilized as nitrogen source, the latter as
193	Utilization of PR digests was investigated in a basal medium (hereinafter referred to as BM) where
192	2.5 Pure culture conditions
191	
190	culturing bifidobacteria, MRS broth was supplemented with 0.5 g/L cysteine hydrochloride.
189	were routinely cultured anaerobically at 37°C in Lactobacilli MRS broth (BD Difco, Sparks, NV, USA). For
188	from the collection of Dept. of Food Science (University of Parma, Italy). Bifidobacteria and lactobacilli
187	Bologna, Italy), or the American Type Culture Collection (ATCC, USA). Lactobacillus strains were obtained
186	of Modena and Reggio Emilia, Italy), the Scardovi Collection of Bifidobacteria (Buscob, University of
185	Bifidobacterium strains were obtained from the collection of the Dept. of Life Sciences (University
184	2.4 Strains and culture conditions
183	
182	software (4.0) and semi-quantified using Phe-Phe area in order to compare relative peptide intensities.
181	Scan mode, with a scan range of 100-2000 m/z . The ions of interest were integrated using MassLynx
180	temperature 350°C; cone gas flow 100 L/h; desolvation gas flow 650 L/h. Samples were analyzed in the Full
179	positive ion mode; capillary voltage 3.2 kV; cone voltage 30 V; source temperature 150°C; desolvation
178	acquisition time 0-58.2 min for water soluble extracts and 7-58.2 min for digested samples; ionization type:
177	sample temperature 18°C; injection volume 10 µL for water soluble extracts and 2 µL for digested samples;

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 diluted 1:100 in Ringer's solution (Oxoid, Basingstoke, UK), then 0.1 ml were used to inoculate tubes 204 containing 6 mL of BM media. The impedance measurement was performed at 37°C. Bacteria growth was 205 measured as E%, where E-value is the impedance change revealed by the system at the electrode surface. 206 207 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 microbial growth. 209

210

211 2.6 Cultures of intestinal microbiota

Batch cultures of fecal microbiota were performed in bioreactors containing 200 mL of FM medium, 212 developed from the media described by Walker et al. (2005) and Duncan et al. (2002), with some 213 modifications: beech wood xylan, 0.6 g/L; citrus pectin, 0.6 g/L; maize amylopectin, 0.6 g/L; larch wood 214 arabinogalactan, 0.6 g/L; potato starch, 3 g/L; fructans (Synergy1, Beneo, Mannheim, Germany), 3 g/L; 215 216 peptone, 5 g/L; ammonium citrate, 2 g/L; KH₂PO₄, 2 g/L; NaCl, 4.5 g/L; MgSO₄ · 7H₂O 0.5 g/L; CaCl₂ · 2H₂O 0.045 g/L; FeSO₄ · 7H₂O, 0.005 g/L; hemin, 0.01 g/L; bile salts (Oxgall, BD Difco) 0.05 g/L, 217 resazurin, 0.6 mg/L; reducing solution, 40 ml/L; mineral solution, 2 ml/L; vitamin solution, 1.4 ml/L. 218 219 Reducing solution contained: cysteine hydrochloride, 12.5 g/L; NaHCO₃, 80 g/L. Mineral solution contained: EDTA, 500 mg/L; FeSO₄ · 7H₂O, 200 mg/L; ZnSO₄ · 7H₂O, 10 mg/L; MnCl₂ · 7H₂O, 3 mg/L; 220 H₃BO₃, 30 mg/L; CoCl₂ · 6H₂O, 20 mg/L; CuCl₂ · 2H₂O, 1 mg/L; NiCl₂ · 6H₂O, 2 mg/L; NaMoO₄ · 2H₂O, 3 221 mg/L. Vitamin solution contained: menadione, 1 g/L; biotin 2 g/L; calcium pantothenate 2 g/L; nicotinamide, 222 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 223 224 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

feces were homogenized (5% w/v) with the medium in the anaerobic cabinet (Anaerobic System, Forma 227 228 Scientific, Marietta, OH) under a 85% N₂, 10% CO₂, 5% H₂ atmosphere. 10 mL of the suspension were inoculated into bioreactors (Sixfors V3.01, Infors, Bottmingen, Switzerland) each containing 190 mL of 229 fresh sterile medium, and the processes proceeded for 48 h (37°C, CO₂ atmosphere, pH 6.2, stirring 60 rpm). 230 5 mL samples were collected at 0, 6, 12, 24 h and stored at -20°C until analyzed. 231 The abundance of total eubacteria, bifidobacteria and lactobacilli was determined by qPCR 232 with the following primer pairs: F-Eub/R-Eub (TCCTACGGGAGGCAGCAGT/ 233 GGACTACCAGGGTATCTAATCCTGTT) (Nadkarni et al., 2002), BiTOT-F/BiTOT-R 234 235 (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

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

calculating the PCA models, the variables were pre-processed using mean centering. The number of

significant PCs, i.e., the number of PCs bearing useful information, was selected based on the scree plot

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 after the *in vitro* simulated gastrointestinal digestion (Figure 1A, 1B). Intact caseins are not taken into 259 260 account, since they were removed from both the water soluble extracts and from the digested samples. Before the simulated digestion, a common peptide profile was detected in the water extracts of PR16, PR24, 261 and PR36 samples (Fig. 1A). 63 main peptides were identified, including some N-lactosyl amino acids, with 262 263 a length up to 103 residues and a molecular weight spread over a wide range (Supporting Information: Table S1). 31 peptides had a MW < 500 Da, 2 had a MW of 500-1000 Da, 12 fell in the range 1000-2000 Da. 264 Longer peptides were also identified, 12 with MW of 2-5 kDa and at 6 with MW of 5-20 kDa. Moreover, the 265 266 two isoforms of β -lactoglobulin (A and B) were clearly detectable at 40.14 min. After *in vitro* digestion, the mixtures of PR16, PR24, and PR36 still shared similar profile. The 267 digests lacked intact proteins, including β -lactoglobulins, and 61 out of the 63 peptides identified in water 268 269 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 water soluble extract, especially of the PR36, and remained in similar amounts after digestion, whereas the 271 latter occurred in the digests in minor amount. N-lactosyl amino acids also resisted digestion, but their 272 273 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

undigested water extracts, molecular weight distribution of the peptides was shifted toward lower MW: 12

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

280

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

Twenty seven *Bifidobacterium* strains belonging to the species *B. adolescentis*, *B. animalis* subsp. 282 283 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 L. rhamnosus were investigated for the ability to grow utilizing PR hydrolysates as nitrogen source. All the 285 strains grew both in the control medium and the PR-based media (Fig. 2), exhibiting a species specific 286 287 growth yield, with few exceptions. Within the phylogenetically related group of *B. adolescentis* and *B. pseudocatenulatum*, the strains belonging to the former species gave similar yields in the diverse BM media, 288 289 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

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

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

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

Bifidobacteria presented different patterns of utilization (Fig. 3). All the strains nearly depleted P1,

P27, P28, P30, and P45 while they did not consume, or scarcely consumed, P39, P53, P69, and P70.

However, preferential utilization or non-utilization of most peptides was associated to one or a group of

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

basis of their peptide preferences observable in the corresponding loading plot (Fig. 5b). *B. adolescentis*

RBB4 (B1), located distant from other bifidobacteria along PC1 (Fig. 5a), was the most efficient in utilizing

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).

B. animalis subsp. *animalis* ATCC 27536 (B3) and *B. animalis* subsp. *lactis* WC 0413 (B4) were the less efficient and grouped together in the PC1-PC2 score plot (Fig. 5A). They consumed only 8-10 peptides for more than 75%, none of them for more than 95% (Fig. 3). The strains of *B. animalis* strains consumed 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).

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

B. longum strains (B7 and B8) shared a similar behavior towards many peptides, but B. longum 332 subsp. infantis WC 0438 (B7) was able to consume also P2, P7, P23, P52, P57, P59, P62, P63, P64, and P71. 333 This explains the strains not grouping together, with a clear separation along PC1, PC2, (Fig. 5a) and PC4 334 335 (Supporting Information: Fig. S1). Peptide consumption by *B. longum* subsp. *longum* WC 0438 (B8) and *B.* pseudocatenulatum WC 0403 (B2) was very similar, being largely overlapped in the first three principal 336 337 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. paracasei 2306 (L7) (Fig. 5c) that clustered separately along PC1 and were characterized by high 343 344 consumption of P15, P46, P49, P57, and P64 and by poor utilization of P13, P27, P68, and P72 (Fig. 5d). P68 and P72 were consumed for more than 60% by all the lactobacilli, with the exception of L. casei 2414 345 346 (L6) and *L. paracasei* 2306 (L7). All the other lactobacilli clustered together at high values of PC1 and presented a similar pattern of 347 peptide utilization. L. rhamnosus 2232 (L1), L. rhamnosus 2325 (L2), L. rhamnosus 2362 (L3), L. casei 2240 348

349 (L4), and L. casei 2404 (L5) are distributed along PC2. L. rhamnosus 2232 (L1) lies at positive values of

PC2, while L. rhamnosus 2325 (L2) and L. casei 2404 (L4) are found at negative PC2 values (Fig. 5c). In

fact, despite their pattern of utilization was very similar, *L. rhamnosus* 2232 (L1) was the most efficient in

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

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

362

363 *3.4 Microbiota cultures*

Cultures of intestinal microbiota were performed in a medium containing 5 g/L of PR24 digest as 364 only nitrogen source or peptone as control (Fig. 6). In both the cultures, total bacteria similarly increased by 365 366 more than 2 magnitudes in the first 6 h of incubation, then remained stationary at a level of approx. 10 Log_{10} cells/mL (Fig. 6A). Bifidobacteria were 6.4 Log₁₀ cells/mL at the beginning of batch process, accounting for 367 4-5% of total bacteria. They grew abundantly in the first 6 h, up to 8.7 and 8.4 Log_{10} cells/mL in PR24 and 368 369 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_{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

The present study aimed to characterize the peptides that are expected to escape digestion of PR and 377 378 to investigate whether they could support the growth of beneficial bacteria in the colon. The water soluble extracts of PR contained a few intact proteins (e.g. the two isoforms of β-lactoglobulin) and a variety of 379 peptides of 2 to 103 residues originated during ripening through the proteolytic cleavage of cheese proteins 380 (mainly α and β -casein) by rennet, SLAB, and NSLAB (McSweeney, 2004). The water extracts of PR16, 381 PR24, and PR36 shared similar profile, consistently with previous evidence that the most relevant 382 383 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 a thorough characterization of the whole profile. The main peptide composition of PR after simulated 387 digestion is reported herein for the first time. The PR digests lacked serum proteins and any peptide longer 388 389 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 α - and β -case in and whey proteins. Any predictable and specific cleavage sites could be found in peptide sequences, consistently with the fact that the 391 392 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.

Some peptides identified in the digests bear sequences of biological interest. Nonetheless, it is not clear whether they could be effectively active, since the sequences were found within longer peptides for which the biological activity has not been investigated so far. In particular, the sequences of the opioids β casomorphin 5 (YPFPG) and β -casomorphin 7 (YPFPGPI) and the ACE inhibitor peptide β -casomorphin 9 (YPFPGPIPN) were found within longer peptides originating from β -casein, such as P50, P56, and P58 (Nguyen et al, 2015). The sequences of the ACE inhibitor tripeptides VPP and IPP were similarly found within longer peptides of the digests, such as P63, P69, and P70. The ACE inhibitor sequence α_{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*.

Potential health promoting effects of PR peptides are supported by observation that the digests of PR 404 sustained abundant growth of pure cultures of bifidobacteria and lactobacilli, natural beneficial colonizers of 405 the human colon and potential probiotics (Rossi and Amaretti, 2010; Walter, 2008). Bifidobacteria were 406 more efficient in consuming PR peptides than lactobacilli, with a greater number of peptides consumed at a 407 greater extent. Peptide preferences of bifidobacteria were specific for each strain, as evidenced by PCA, with 408 a core of peptides with 4 or 5 residues consumed by all strains (P1, P27, P28, P30, and P45). On the other 409 410 hand, lactobacilli behaved very homogenously 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 bifidobacteria and lactobacilli could not be ascribed to features such as the length of the peptide, the presence 417 of sulfur-containing amino acids, or the abundance of residues with peculiar properties (hydrophobicity, 418 419 polarity, charge) and likely depend on specific proteases and/or peptide transporters preferentially recognizing specific sequence motifs in the peptides. 420 The bifidogenic effect of PR digests in pure and microbiota cultures is coherent with previous 421 studies which described the stimulation of bifidobacteria by peptic and/or tryptic digests of certain milk 422

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 Log10 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
- 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
- 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
- 446 Acknowledgement
- 447 The study was funded by Project SIME2013.0627 (Fondazione Cassa di Risparmio di Modena, Italy).
- 448
- 449 5 References
- 450 Alessandri, C., Sforza, S., Palazzo, P., Lambertini, F., Paolella, S., Zennaro, D., Rafaiani, C., Ferrara, R.,
- 451 Bernardi, M. L., Santoro, M., Zuzzi, S., Giangrieco, I., Dossena, A., Mari, A., 2012. Tolerability of a

- 452 fully maturated cheese in cow's milk allergic children: biochemical, immunochemical, and clinical
 453 aspects. PLoS One 7, e40945.
- 454 Bancalari, E., Bernini, V., Bottari, B., Neviani, E., Gatti, M., 2016. Application of impedance microbiology
- 455 for evaluating potential acidifying performances of starter lactic acid bacteria to employ in milk
- 456 transformation. Front. Microbiol. 7:1628
- 457 Basiricò, L., Catalani, E., Morera, P., Cattaneo, S., Stuknytė, M., Bernabucci, U., De Noni, I., Nardone, A.,
- 458 2015. Release of angiotensin converting enzyme-inhibitor peptides during *in vitro* gastrointestinal
- digestion of Parmigiano Reggiano PDO cheese and their absorption through an *in vitro* model of
- 460 intestinal epithelium. J. Dairy Sci. 98, 7595-7601.
- Benkerroum, N., 2010. Antimicrobial peptides generated from milk proteins: a survey and prospects for
 application in the food industry. A review. Int. J. Dairy Tech. 63, 320-338.
- 463 Bernabucci, U., Catalani, E., Basiricò, L., Morera, P., Nardone, A., 2014. In vitro ACE-inhibitory activity
- and *in vivo* antihypertensive effects of water-soluble extract by Parmigiano Reggiano and Grana Padano
 cheeses. Int. Dairy J. 37, 16-19.
- 466 Bordoni, A., Picone, G., Babini, E., Vignali, M., Danesi, F., Valli, V., Di Nunzio, M., Laghi, L., Capozzi, F.,
- 467 2011. NMR comparison of in vitro digestion of Parmigiano Reggiano cheese aged 15 and 30 months.
- 468 Magn. Reson. Chem. 49, S61-S70.
- 469 Bottesini, C., Paolella, S., Lambertini, F., Galaverna, G., Tedeschi, T., Dossena, A., Marchelli, R., Sforza, S.,
- 470 2013. Antioxidant capacity of water soluble extracts from Parmigiano-Reggiano cheese. Int. J. Food Sci.
 471 Nutr. 64, 953-958.
- 472 Castillo, M., Martín-Orúe, S.M., Manzanilla, E.G., Badiola, I., Martín, M., Gasa, J., 2006. Quantification of
- total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. Vet. Microbiol.
- 474 114, 165-70.
- 475 Condezo-Hoyos, L., Noratto, G., 2016. Aged American cheddar cheese as source of protein derived
- 476 compounds that modulate obese mice fecal bacteria and colon inflammation *in vitro*. Symbiosis. 4(5), 1-

477 <u>10.</u>

- 478 De Noni, I., Cattaneo, S., 2010. Occurrence of b-casomorphins 5 and 7 in commercial dairy products and in
 479 their digests following in vitro simulated gastro-intestinal digestion. Food Chem. 119, 560–566.
- 480 Duncan, S.H., Barcenilla, A., Stewart, C.S., Pryde, S.E., Flint, H.J., 2002. Acetate utilization and butyryl
- 481 coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large
- 482 intestine. Appl. Environ. Microbiol. 68, 5186-5190.
- 483 European Commission, 2009. Amendment application to Council Regulation (EC) No 510/2006 pursuant to
- 484 Article 9 'PARMIGIANO REGGIANO' EC No: IT-PDO-0317-0016-26.7.2007. Official J. Europ.
 485 Union. C87/06, 14-19.
- 486 European Commission, 2011. Commission Regulation (EU) No 794/2011 approving amendments to the
- 487 specification for a name entered in the register of protected designations of origin and protected
- 488 geographical indications (Parmigiano Reggiano (PDO)). Official J. Europ. Union. L204, 19-20.
- Fox, P.F., McSweeney, P.L.H., 1998. Dairy Chemistry and Biochemistry. Blackie Academic and
 Professional Publishers, London.
- 491 Gatti, M., Bottari, B., Lazzi, C., Neviani, E., Mucchetti, G., 2014. Invited review: Microbial evolution in
- raw-milk, long-ripened cheeses produced using undefined natural whey starters. J Dairy Sci. 97, 573-591.
- Gupta, A., Mann, B., Kumar, R., Sangwan, R., 2009. Antioxidant activity of Cheddar cheeses at different
 stages of ripening. Int. J. Dairy Tech. 62, 339-347.
- 495 Hernàndez-Ledesma, B., Martìnez-Maqueda, D., Miralles, B., Amigo, L., Gòmez-Ruiz, J.Á. 2012. *Peptides*.
- 496 In: Nollet, L.M.L., Toldrà, F. (Eds.) Food Analysis by HPLC, Third Edition. CRC Press, Boca Raton,
- 497 USA, pp. 69-95.
- Ibrahim, S. A. Bezkorovainy, A., 1994. Growth-promoting factors for *Bifidobacterium longum*. J. Food. Sc.
 59, 189–191.
- 500 Kieronczyk, A., Skeie, S., Olsen K., Langsrud, T., 2001. Metabolism of amino acids by resting cells of non-
- starter lactobacilli in relation to flavour development in cheese. Intern. Dairy J. 11, 217-224.

- Kim, S. B., Lim, J. W., 2004. Calcium-binding peptides derived from tryptic hydrolysates of cheese whey
 protein. Asian-Aust. J. Anim. Sci. 17, 1459-1464.
- Korhonen, H., 2009. Milk-derived bioactive peptides: from science to applications. J. Funct. Foods 1, 177187.
- Liepke, C., Adermann, K., Raida, M., Mägert, H. J., Forssmann, W. G., Zucht, H. D., 2002. Human milk
 provides peptides highly stimulating the growth of Bifidobacteria. Eur. J. Biochem. 269, 712-718.
- 508 Massart, D.L., Vandenginste, B.G.M., Buydens, I.M.C., De Jong S., Lewi P.J., Smeyers-Verbeke J., 1997.
- 509 Handbook of Chemometrics and Qualimetrics: Part A. Elsevier, Amsterdam, pp. 519-556.
- 510 McSweeney, P.L.H., 2004. Biochemistry of cheese ripening. Int. J. Dairy Tech. 57, 127-144.
- Meli, F., Lazzi, C., Neviani, E., Gatti, M., 2013. Effect of protein hydrolizates on growth kinetics and
 aminopeptidase activities of some *Bifidobacterium* species. Anaerobe 22, 130-133.
- Meli, F., Lazzi, C., Neviani, E., Gatti, M., 2014. Effect of protein hydrolysates on growth kinetics and
 aminopeptidase activities of *Lactobacillus*. Curr. Microbiol. 68, 82-87.
- 515 Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, Corredig,
- 516 M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes,
- 517 U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., M'enard, O., Recio, I., Santos, C.N.,
- 518 Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W., Brodkorb, A., 2014. A standardised static
- *in vitro* digestion method suitable for food an international consensus. Food Funct. 5, 1113–1124.
- 520 Nadkarni, M.A., Martin, F.E., Jacques, N.A., Hunter, N., 2002. Determination of bacterial load by real-time
- 521 PCR using a broad-range (universal) probe and primers set. Microbiology 148, 257-266.
- 522 Nguyen, D.D., Johnson, S.K., Busetti, F., Solah, V.A., 2015. Formation and Degradation of Beta-
- 523 casomorphins in Dairy Processing. Crit. Rev. Food Sci. Nutr. 55, 1955-1967.
- 524 Oda, H., Wakabayashi, H., Yamauchi, K., Sato, T., Xiao, J.Z., Abe, F., Iwatsuki, K. 2013. Isolation of a
- 525 bifidogenic peptide from the pepsin hydrolysate of bovine lactoferrin. Appl Environ Microbiol. 79, 1843-
- 526 <u>1849.</u>

- 527 Olivi, O., Balli, F., Crema, G., Squadrini, F., Taparelli, F., 1979. Changes induced by diet therapy with
- casein hydrolysate on plasmatic free amino acids in infants with viral enteritis. Minerva Pediatr. 31, 14591467.
- Pancaldi, M., Mariotti, I., Balli, F., 2008. Intestinal inflammation in nursing infants: different causes and a
 single treatment... but of protected origin. Acta Biomed. 79, 144-150.
- 532 Pinto, G., Caira, S., Cuollo, M., Lilla, S., Chianese, L., Addeo, F., 2012. *Bioactive casein phosphopeptides in*
- 533 *dairy products as nutraceuticals for functional foods.* In: Hurley, W.L. (Ed.), Milk Protein. Intech,
- 534 Rijeka, pp. 3-44.
- Poch, M., Bezkorovainy, A., 1991. Bovine milk k-casein trypsin digest is a growth enhancer for the genus *Bifidobacterium*. J. Agric. Food Chem. 39, 73-77.
- 537 Quartieri, A., Simone, M., Gozzoli, C., Popovic, M., D'Auria, G., Amaretti, A., Raimondi, S., Rossi, M.,
- 538 2016. Comparison of culture-dependent and independent approaches to characterize fecal bifidobacteria539 and lactobacilli. Anaerobe 38, 130-137.
- 540 Richardson, A.J., McKain, N., Wallace, R.J., 2013. Ammonia production by human faecal bacteria, and the
- enumeration, isolation and characterization of bacteria capable of growth on peptides and amino acids.
- 542 BMC Microbiol. 13, 6.
- 543 Rinttilä, T., Kassinen, A., Malinen, E., Krogius, L., Palva, A.J., 2004. Development of an extensive set of
- 544 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by
- 545 real-time PCR. Appl. Microbiol. 97, 1166-1177.
- 546 Rizzello, C.G., Losito, I., Gobbetti, M., Carbonara, T., De Bari, M. D., Zambonin, P.G., 2005. Antibacterial
- activities of peptides from the water-soluble extracts of Italian cheese varieties. J. Dairy Sci. 88, 23482360.
- 549 Rossi, M., Amaretti, A., 2010. Probiotic properties of bifidobacteria. In: van Synderen, D., Mayo, B. (Eds.),
- 550 Bifidobacteria: Genomics and Molecular Aspects. Horizon Scientific Press, Rowan House, UK, pp. 97-
- 551 123.

- 552 Rossi, M., Martínez-Martínez, D., Amaretti, A., Ulrici, A., Raimondi, S., Moya, A., 2016. Mining
- 553 metagenomic whole genome sequences revealed subdominant but constant *Lactobacillus* population in
- the human gut microbiota. *Environ. Microbiol. Rep.* 8, 399-406.
- Sekirov, I., Russell, S.L., Antunes, L.C., Finlay, B.B., 2010. Gut microbiota in health and disease. Physiol.
 Rev. 90, 859-904.
- Sforza, S., Cavatorta, V., Galaverna, G., Dossena, A., Marchelli, R., 2009. Accumulation of non-proteolytic
 aminoacyl derivatives in Parmigiano-Reggiano cheese during ripening. Int. Dairy J. 19, 582-587.
- 559 Sforza, S., Cavatorta, V., Lambertini, F., Galaverna, G., Marchelli, R., 2012. Cheese peptidomics: a detailed
- study on the evolution of the oligopeptide fraction in Parmigiano-Reggiano cheese from curd to 24
- 561 months of aging. J. Dairy Sci. 95, 3514-3526.
- 562 Sgarbi E., Bottari B., Gatti M., Neviani, E., 2014. Investigation of the ability of dairy nonstarter lactic acid
- bacteria to grow using cell lysates of other lactic acid bacteria as the exclusive source of nutrients. Int. J.
 Dairy Technol. 67, 342–347.
- 565 Simone, M., Gozzoli, C., Quartieri, A., Mazzola, G., Di Gioia, D., Amaretti, A., Raimondi, S., Rossi, M.,
- 566 2014. The probiotic *Bifidobacterium breve* B632 inhibited the growth of *Enterobacteriaceae* within
- colicky infant microbiota cultures. Biomed. Res. Int. 2014, 301053.
- Tidona, F., Criscione, A., Guastella, A.M., Zuccaro, A., Bordonaro, S., Marletta, D., 2009. Bioactive
 peptides in dairy products. Ital. J. Anim. Sci. 8, 315-340.
- 570 Visser, S., 1993. Proteolytic enzymes and their relation to cheese ripening and flavor: an overview. J. Dairy
 571 Sci. 76, 329-350.
- 572 Walker, A.W., Duncan, S.H., McWilliam Leitch, E.C., Child, M.W., Flint, H.J., 2005. pH and peptide supply
- 573 can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities
- from the human colon. Appl. Environ. Microbiol. 71, 3692-3700.
- Walter, J., 2008. Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and
 biomedical research. Appl. Environ. Microbiol. 74, 4985-4996.

577 Wold, S., Esbensen, K., Geladi, P., 1987. Principal component analysis. Chemom. Intell. Lab. Syst. 2, 37-52.

578 Figure legends

- 579 Figure 1. Overlaid Full Scan chromatograms obtained for the waters soluble extracts (Panel A) and the
- 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
- 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 (OD₆₀₀) and
- impedance (E%), respectively. Values are means, n = 3, SD < 0.1. The box and whiskers plots (Panel C)
- report, for each species or group of species/subspecies, the increase in the different media. Boxes indicate the
- median and 25^{th} and 75^{th} percentiles; whiskers indicate the minimum and the maximum.
- 588 Figure 3. Peptide consumption by *Bifidobacterium* and *Lactobacillus* strains cultured for 24 h in BM
- 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
- (deepest green) to the highest (deepest red) consumption. Values are means, n = 3, SD always < 7.
- Figure 4. PCA model of peptide consumption by *Bifidobacterium* and *Lactobacillus* strains: PC1-PC2 score
 plot (a) and loading plot (b).
- **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,
- dashed line; relative amount of bifidobacteria in the microbiota, solid line. Values are means \pm 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

- 3 Benedetta Bottari^b, Andrea Quartieri^a; Barbara Prandi^b; Stefano Raimondi^a; Alan Leonardi^a; Maddalena
- 4 Rossi^{a,c}, Alessandro Ulrici^{a,c}; Monica Gatti^{b,d}; Stefano Sforza^b; Marco Nocetti^e; Alberto Amaretti^{a,c}
- ^a Department of Life Sciences, University of Modena and Reggio Emilia, Italy
- 6 ^b Department of Food Science, University of Parma, Italy
- ^c Biogest-Siteia, Centro per il Miglioramento e la Valorizzazione delle Risorse Biologiche Agro-Alimentari,
- 8 University of Modena and Reggio Emilia, Italy
- 9 ^d Multidisciplinary Interdepartmental Dairy Center MILC, University of Parma, Parma, Italy
- 10 ^e Servizio Tecnico Consorzio del Formaggio Parmigiano Reggiano, Reggio Emilia, Italy

11

- 12 Correspondence: Alberto Amaretti, PhD, Dept. Life Sciences, University of Modena and Reggio Emilia,
- 13 Via G. Campi 103, 41125 Modena, Italy, alberto.amaretti@unimore.it

15 Abstract

16 Parmigiano Reggiano (PR) is a raw-milk, hard cooked, long-ripened cheese of high quality and nutritional value. Long ripening times allow for extensive proteolysis of milk proteins to yield a number of peptides, 17 some of which have potential healthy bioactive properties. This study aimed to: i) determine the peptide 18 profile of PR cheese subjected to simulated gastrointestinal transit; ii) evaluate in vitro whether the peptides 19 could support growth of beneficial microbial groups of the gut microbiota. PR samples were subjected to in 20 vitro digestion, simulating oral, gastric, and duodenal transit. Liquid chromatography coupled with tandem 21 mass spectrometry revealed that digestion caused the disappearance of the serum proteins and most of the 22 23 original peptides, while 71 new peptides were found, all ranging from 2 to 24 residues. The digests were given as sole nitrogen source to pure cultures of *Bifidobacterium* (27 strains) and *Lactobacillus* (30 strains), 24 and to bioreactor batch cultures of human gut microbiota. Most of bifidobacteria and lactobacilli grew more 25 abundantly on PR digests than on the control peptone, and exhibited strain- or species- specific peptide 26 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 homogenously 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 abundance of residues with peculiar properties (hydrophobicity, polarity, charge) and likely depend on 33 34 specific proteases and/or peptide transporters preferentially recognizing specific sequence motifs. The cultures of human colonic microbiota confirmed that PR digest promoted the growth of commensal 35 bifidobacteria. This study demonstrated that peptides derived from simulated gastrointestinal digestion of PR 36 supported the growth of most lactobacilli and bifidobacteria. 37

38

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

1 Introduction 52

53 Parmigiano Reggiano (PR) is a raw-milk, hard cooked, long-ripened cheese of high quality and nutritional value, produced in a restricted area in northern Italy. PR is produced from bovine milk with rennet 54 and natural whey starter and a Protected Designation of Origin, in compliance with the European norm 55 currently in force (European Commission, 2009, 2011). The curd is heated at 55°C to select thermophilic 56 57 bacterial strains and it is ripened at least for 12 months, even if much longer ageing times are usually adopted. In mature PR, the moisture is 28 to 35%. The dry weight is mostly composed of proteins and lipids 58 fractions, the ratio of fat to protein being around 0.94, depending on milk characteristics. Ripened PR is 59 60 lactose- and galactose-free and rich in free organic acids, mostly derived from bacterial fermentations, such as lactic acid (1.5 g per 100 g of PR), citric (50 mg), acetic (100 mg), propionic (0.5 mg), and butyric (120 61 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 aminoacyl derivatives also accumulate (Sforza et al., 2009, 2012). Casein breakdown contributes to 68 improved digestibility, reduced allergenicity, and flavor development (Alessandri et al., 2012; Sforza et al., 69 2012). The potential biological activity of PR peptides has recently attracted particular interest (Korhonen et 70 al., 2009; Tidona et al., 2009). In vitro studies revealed calcium binding properties (Kim et al., 2004; Pinto et 71 al., 2012) and antioxidant (Bottesini et al. 2013; Gupta et al., 2009), antihypertensive (Bernabucci et al., 72 73 2014), antimicrobial activities (Benkerroum, 2010; Rizzello et al., 2005)... The nutritional value of PR, coupled with the potential beneficial properties of bioactive peptides 74

that could impact health, suggested its use as functional food in a dietary therapy for subjects with 75 76

inflammatory GIT diseases resulting from alimentary intolerance, post-therapeutic antibiotic-associated

dismicrobism, or post-infective conditions (Olivi et al., 1979; Pancaldi et al., 2008). In particular, a 77 78 homemade food based on PR cheese was developed and successfully utilized to feed infants suffering from different forms of intestinal problems, with a rapid clinical improvement and normalization of the gut. The 79 high digestibility and the high amounts of short chain fatty acids, amino acids, and oligopeptides easily 80 absorbed in the bowel likely support this beneficial effect. Furthermore, the hypoallergenicity, the absence of 81 lactose, and the high sodium content that restores losses of salts and reduces the secretions of intestinal cells 82 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 effects on the health status (Sekirov et al., 2010), wherein the commensal bifidobacteria and lactobacilli are 86 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 Bezkorovainy, 1994; Liepke et al., 2002; Oda et al., 2013; Meli et al., 2013, 2014). Moreover, the peptides 90 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). The health effects of PR bioactive peptides on intestinal bacteria require that they resist 95

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 910 specifically promoting the growth of beneficial bacteria and potential probiotics could be identified, the 92 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*

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

114

115 *2.1 Simulated gastrointestinal digestion of PR samples*

All the chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) unless otherwise 116 stated. The cheese was finely grounded and digested following the procedure, consisting in three main steps: 117 118 salivary phase, gastric phase, and intestinal phase (Minekus et al., 2014). A sample of 25 g cheese was added to 17.5 mL of salivary buffer (15.1 mM KCl, 3.7 mM KH₂PO₄, 13.6 mM NaHCO₃, 0.15 mM MgCl₂, and 119 0.06 mM (NH₄)₂CO₃), 2.5 mL of 1500 U/mL amylase, 125 µL of 300 mM CaCl₂, and 4.875 mL of distilled 120 water (ratio cheese:digestive fluid of 1:1, w:v). The sample was vortexed and incubated for 2 min at 37°C on 121 a reciprocating shaker. Then, 37.5 mL of gastric buffer (6.9 mM KCl, 0.9 mM KH₂PO₄, 25 mM NaHCO₃, 122 47.2 mM NaCl, 0.1 mM MgCl₂, and 0.5 mM (NH₄)₂CO₃), 8 mL of 25000 U/mL pepsin, 25 µL of 300 mM 123 CaCl₂, 1 mL of 1 M HCl and 3.475 mL of water were added (final ratio cheese:digestive fluids 1:3, w:v). 124 125 The pH was adjusted to 3 with 1 M HCl. The mixture was vortexed and incubated for 2 h at 37°C on a reciprocating shaker. Finally, 55 mL of intestinal buffer (6.8 mM KCl, 0.8 mM KH₂PO₄, 85 mM NaHCO₃, 126

127	38.4 mM NaCl, 0.33 mM MgCl ₂), 25 mL of 800 U/mL pancreatin, 12.5 mL of 75 mg/mL bile solution, 200
128	μL of 300 mM CaCl_2, 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 \times g to remove insoluble proteins and
133	undigested components (Minekus et al., 2014). The supernatant was filtered through 0.45 μ m membranes.
134	For chromatographic analysis, 196 μ L of sample was supplemented with 4 μ 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 μ m
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 μ 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.

152Peptides were manually identified as follows. Molecular ions were recorded from the Full Scan153chromatogram, then they were fragmented in Daughter Scan modality using the triple quadrupole equipment.154The MW of each peptide was launched in FindPept tool (http://web.expasy.org/findpept last accessed on155September 2016) against the main milk proteins (α-casein, β-casein, κ-casein, α-lactalbumin and β-156lactoglobulin), to obtain a list of peptide sequences with a compatible MW. The theoretical fragmentation157pattern of these sequences was determined158(http://db.systemsbiology.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 matching160 fragments.

161

162 2.3 UPLC/ESI-MS analysis.

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

177	sample temperature 18°C; injection volume 10 μ L for water soluble extracts and 2 μ L 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 ₂ HPO ₄ , 2 g/l; MgSO ₄ \cdot 7H ₂ O, 0.1 g/l; MnSO ₄ \cdot
198	H_2O , 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_{600})

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 diluted 1:100 in Ringer's solution (Oxoid, Basingstoke, UK), then 0.1 ml were used to inoculate tubes 204 containing 6 mL of BM media. The impedance measurement was performed at 37°C. Bacteria growth was 205 measured as E%, where E-value is the impedance change revealed by the system at the electrode surface. 206 207 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 microbial growth. 209

210

211 2.6 Cultures of intestinal microbiota

Batch cultures of fecal microbiota were performed in bioreactors containing 200 mL of FM medium, 212 developed from the media described by Walker et al. (2005) and Duncan et al. (2002), with some 213 modifications: beech wood xylan, 0.6 g/L; citrus pectin, 0.6 g/L; maize amylopectin, 0.6 g/L; larch wood 214 arabinogalactan, 0.6 g/L; potato starch, 3 g/L; fructans (Synergy1, Beneo, Mannheim, Germany), 3 g/L; 215 216 peptone, 5 g/L; ammonium citrate, 2 g/L; KH₂PO₄, 2 g/L; NaCl, 4.5 g/L; MgSO₄ · 7H₂O 0.5 g/L; CaCl₂ · 2H₂O 0.045 g/L; FeSO₄ · 7H₂O, 0.005 g/L; hemin, 0.01 g/L; bile salts (Oxgall, BD Difco) 0.05 g/L, 217 resazurin, 0.6 mg/L; reducing solution, 40 ml/L; mineral solution, 2 ml/L; vitamin solution, 1.4 ml/L. 218 219 Reducing solution contained: cysteine hydrochloride, 12.5 g/L; NaHCO₃, 80 g/L. Mineral solution 220 contained: EDTA, 500 mg/L; FeSO₄ · 7H₂O, 200 mg/L; ZnSO₄ · 7H₂O, 10 mg/L; MnCl₂ · 7H₂O, 3 mg/L; H₃BO₃, 30 mg/L; CoCl₂ · 6H₂O, 20 mg/L; CuCl₂ · 2H₂O, 1 mg/L; NiCl₂ · 6H₂O, 2 mg/L; NaMoO₄ · 2H₂O, 3 221 mg/L. Vitamin solution contained: menadione, 1 g/L; biotin 2 g/L; calcium pantothenate 2 g/L; nicotinamide, 222 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 223 224 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

feces were homogenized (5% w/v) with the medium in the anaerobic cabinet (Anaerobic System, Forma 227 228 Scientific, Marietta, OH) under a 85% N₂, 10% CO₂, 5% H₂ atmosphere. 10 mL of the suspension were inoculated into bioreactors (Sixfors V3.01, Infors, Bottmingen, Switzerland) each containing 190 mL of 229 fresh sterile medium, and the processes proceeded for 48 h (37°C, CO₂ atmosphere, pH 6.2, stirring 60 rpm). 230 5 mL samples were collected at 0, 6, 12, 24 h and stored at -20°C until analyzed. 231 The abundance of total eubacteria, bifidobacteria and lactobacilli was determined by qPCR 232 with the following primer pairs: F-Eub/R-Eub (TCCTACGGGAGGCAGCAGT/ 233 GGACTACCAGGGTATCTAATCCTGTT) (Nadkarni et al., 2002), BiTOT-F/BiTOT-R 234 235 (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

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

calculating the PCA models, the variables were pre-processed using mean centering. The number of

significant PCs, i.e., the number of PCs bearing useful information, was selected based on the scree plot

reporting the percentage of variance explained by each PC vs. the PC number.

255

275

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 after the *in vitro* simulated gastrointestinal digestion (Figure 1A, 1B). Intact caseins are not taken into 259 260 account, since they were removed from both the water soluble extracts and from the digested samples. Before the simulated digestion, a common peptide profile was detected in the water extracts of PR16, PR24, 261 and PR36 samples (Fig. 1A). 63 main peptides were identified, including some N-lactosyl amino acids, with 262 263 a length up to 103 residues and a molecular weight spread over a wide range (Supporting Information: Table S1). 31 peptides had a MW < 500 Da, 2 had a MW of 500-1000 Da, 12 fell in the range 1000-2000 Da. 264 Longer peptides were also identified, 12 with MW of 2-5 kDa and at 6 with MW of 5-20 kDa. Moreover, the 265 266 two isoforms of β -lactoglobulin (A and B) were clearly detectable at 40.14 min. After *in vitro* digestion, the mixtures of PR16, PR24, and PR36 still shared similar profile. The 267 digests lacked intact proteins, including β -lactoglobulins, and 61 out of the 63 peptides identified in water 268 269 soluble extracts were no longer detectable, being degraded into shorter ones (Fig. 1B). The sole exception were FVAPFPEVFGK and the phosphopeptide LEELNVPGEIVESLSSS. The former was present in the 270 water soluble extract, especially of the PR36, and remained in similar amounts after digestion, whereas the 271 272 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)

undigested water extracts, molecular weight distribution of the peptides was shifted toward lower MW: 12

and, with the exception of P3, P13, P71, and P72, were assigned to specific sequences. Compared with the

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

280

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

Twenty seven *Bifidobacterium* strains belonging to the species *B. adolescentis*, *B. animalis* subsp. 282 283 animalis, B. animalis subsp. lactis, B. bifidum, B. breve, B. longum subsp. infantis, B. longum subsp. longum, and B. pseudocatenulatum, and 30 Lactobacillus strains belonging to the species L. casei, L. paracasei and 284 L. rhamnosus were investigated for the ability to grow utilizing PR hydrolysates as nitrogen source. All the 285 strains grew both in the control medium and the PR-based media (Fig. 2), exhibiting a species specific 286 growth yield, with few exceptions. Within the phylogenetically related group of *B. adolescentis* and *B.* 287 pseudocatenulatum, the strains belonging to the former species gave similar yields in the diverse BM media, 288 289 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. rhamnosus strains 2323, 2303, 2302, 2409 grew better on the control peptone. A trend common to all the 293 294 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

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

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

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

Bifidobacteria presented different patterns of utilization (Fig. 3). All the strains nearly depleted P1,

P27, P28, P30, and P45 while they did not consume, or scarcely consumed, P39, P53, P69, and P70.

However, preferential utilization or non-utilization of most peptides was associated to one or a group of

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

basis of their peptide preferences observable in the corresponding loading plot (Fig. 5b). *B. adolescentis*

RBB4 (B1), located distant from other bifidobacteria along PC1 (Fig. 5a), was the most efficient in utilizing

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).

B. animalis subsp. *animalis* ATCC 27536 (B3) and *B. animalis* subsp. *lactis* WC 0413 (B4) were the less efficient and grouped together in the PC1-PC2 score plot (Fig. 5A). They consumed only 8-10 peptides for more than 75%, none of them for more than 95% (Fig. 3). The strains of *B. animalis* strains consumed 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).

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

B. longum strains (B7 and B8) shared a similar behavior towards many peptides, but B. longum 332 subsp. infantis WC 0438 (B7) was able to consume also P2, P7, P23, P52, P57, P59, P62, P63, P64, and P71. 333 This explains the strains not grouping together, with a clear separation along PC1, PC2, (Fig. 5a) and PC4 334 335 (Supporting Information: Fig. S1). Peptide consumption by *B. longum* subsp. *longum* WC 0438 (B8) and *B.* pseudocatenulatum WC 0403 (B2) was very similar, being largely overlapped in the first three principal 336 337 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. paracasei 2306 (L7) (Fig. 5c) that clustered separately along PC1 and were characterized by high 343 344 consumption of P15, P46, P49, P57, and P64 and by poor utilization of P13, P27, P68, and P72 (Fig. 5d). P68 and P72 were consumed for more than 60% by all the lactobacilli, with the exception of L. casei 2414 345 346 (L6) and *L. paracasei* 2306 (L7). All the other lactobacilli clustered together at high values of PC1 and presented a similar pattern of 347 peptide utilization. L. rhamnosus 2232 (L1), L. rhamnosus 2325 (L2), L. rhamnosus 2362 (L3), L. casei 2240 348

349 (L4), and L. casei 2404 (L5) are distributed along PC2. L. rhamnosus 2232 (L1) lies at positive values of

PC2, while L. rhamnosus 2325 (L2) and L. casei 2404 (L4) are found at negative PC2 values (Fig. 5c). In

fact, despite their pattern of utilization was very similar, *L. rhamnosus* 2232 (L1) was the most efficient in

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

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

362

363 *3.4 Microbiota cultures*

Cultures of intestinal microbiota were performed in a medium containing 5 g/L of PR24 digest as 364 only nitrogen source or peptone as control (Fig. 6). In both the cultures, total bacteria similarly increased by 365 366 more than 2 magnitudes in the first 6 h of incubation, then remained stationary at a level of approx. 10 Log_{10} cells/mL (Fig. 6A). Bifidobacteria were 6.4 Log₁₀ cells/mL at the beginning of batch process, accounting for 367 4-5% of total bacteria. They grew abundantly in the first 6 h, up to 8.7 and 8.4 Log_{10} cells/mL in PR24 and 368 369 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_{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

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

385 Few previous studies performed in vitro digestion of PR, but mainly focused on quantification of specific peptides (Bordoni et al., 2011; De Noni and Cattaneo, 2010; Basiricò et al., 2015), without providing 386 a thorough characterization of the whole profile. The main peptide composition of PR after simulated 387 digestion is reported herein for the first time. The PR digests lacked serum proteins and any peptide longer 388 389 than 24 residues. The new peptides derived from the hydrolysis of the high molecular weight peptides already present in cheese and from the breakdown of intact α - and β -casein and whey proteins. Any 390 391 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.

Some peptides identified in the digests bear sequences of biological interest. Nonetheless, it is not clear whether they could be effectively active, since the sequences were found within longer peptides for which the biological activity has not been investigated so far. In particular, the sequences of the opioids β casomorphin 5 (YPFPG) and β -casomorphin 7 (YPFPGPI) and the ACE inhibitor peptide β -casomorphin 9 (YPFPGPIPN) were found within longer peptides originating from β -casein, such as P50, P56, and P58 (Nguyen et al, 2015). The sequences of the ACE inhibitor tripeptides VPP and IPP were similarly found within longer peptides of the digests, such as P63, P69, and P70. The ACE inhibitor sequence α_{s1} -cn f(24– 402

403

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

Potential health promoting effects of PR peptides are supported by observation that the digests of PR 404 sustained abundant growth of pure cultures of bifidobacteria and lactobacilli, natural beneficial colonizers of 405 the human colon and potential probiotics (Rossi and Amaretti, 2010; Walter, 2008). Bifidobacteria were 406 more efficient in consuming PR peptides than lactobacilli, with a greater number of peptides consumed at a 407 greater extent. Peptide preferences of bifidobacteria were specific for each strain, as evidenced by PCA, with 408 a core of peptides with 4 or 5 residues consumed by all strains (P1, P27, P28, P30, and P45). On the other 409 410 hand, lactobacilli behaved very homogenously and seemed to prefer the same peptides, mostly di- and tripeptides, such as P4, P9, P12, P18, and P20. The differences among lactobacilli were minor and restricted 411 412 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 clustering bacteria on the basis of peptide utilization, no grouping and/or distribution of the peptides could be 415 416 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 of sulfur-containing amino acids, or the abundance of residues with peculiar properties (hydrophobicity, 418 419 polarity, charge) and likely depend on specific proteases and/or peptide transporters preferentially recognizing specific sequence motifs in the peptides. 420

The bifidogenic effect of PR digests in pure and microbiota cultures is coherent with previous studies which described the stimulation of bifidobacteria by peptic and/or tryptic digests of certain milk proteins (Poch and Bezkorovainy, 1991; Oda et al. 2013; Condezo-Hoyos et al. 2016). In microbiota cultures, the digest of PR24 promoted the growth of bifidobacteria, the counts of which increased in the first hours of fermentation and achieved a higher relative abundance towards the end of the fermentation, 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 Log10 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

The present study describes several new, previously not reported, peptides originating from cheese 434 435 digestion, thus opening the question whether they are bioactive. Determining the bioactivity of the peptides of PR digests is attractive and deserves further studies. Despite the limitation of the experimental procedure, 436 that did not take into account the absorption in the small intestine, it was demonstrated that PR digests 437 438 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 herein presented, opens intriguing perspective in the modulation of the gut microbiota towards a healthy 443 444 composition and deserves deeper investigation.

445

446 Acknowledgement

The study was funded by Project SIME2013.0627 (Fondazione Cassa di Risparmio di Modena, Italy).

449 **5 References**

Alessandri, C., Sforza, S., Palazzo, P., Lambertini, F., Paolella, S., Zennaro, D., Rafaiani, C., Ferrara, R.,
Bernardi, M. L., Santoro, M., Zuzzi, S., Giangrieco, I., Dossena, A., Mari, A., 2012. Tolerability of a

- 452 fully maturated cheese in cow's milk allergic children: biochemical, immunochemical, and clinical
 453 aspects. PLoS One 7, e40945.
- 454 Bancalari, E., Bernini, V., Bottari, B., Neviani, E., Gatti, M., 2016. Application of impedance microbiology
- 455 for evaluating potential acidifying performances of starter lactic acid bacteria to employ in milk
- 456 transformation. Front. Microbiol. 7:1628
- 457 Basiricò, L., Catalani, E., Morera, P., Cattaneo, S., Stuknytė, M., Bernabucci, U., De Noni, I., Nardone, A.,
- 458 2015. Release of angiotensin converting enzyme-inhibitor peptides during *in vitro* gastrointestinal
- digestion of Parmigiano Reggiano PDO cheese and their absorption through an *in vitro* model of
- 460 intestinal epithelium. J. Dairy Sci. 98, 7595-7601.
- Benkerroum, N., 2010. Antimicrobial peptides generated from milk proteins: a survey and prospects for
 application in the food industry. A review. Int. J. Dairy Tech. 63, 320-338.
- 463 Bernabucci, U., Catalani, E., Basiricò, L., Morera, P., Nardone, A., 2014. In vitro ACE-inhibitory activity
- and *in vivo* antihypertensive effects of water-soluble extract by Parmigiano Reggiano and Grana Padano
 cheeses. Int. Dairy J. 37, 16-19.
- 466 Bordoni, A., Picone, G., Babini, E., Vignali, M., Danesi, F., Valli, V., Di Nunzio, M., Laghi, L., Capozzi, F.,
- 467 2011. NMR comparison of in vitro digestion of Parmigiano Reggiano cheese aged 15 and 30 months.
- 468 Magn. Reson. Chem. 49, S61-S70.
- 469 Bottesini, C., Paolella, S., Lambertini, F., Galaverna, G., Tedeschi, T., Dossena, A., Marchelli, R., Sforza, S.,
- 470 2013. Antioxidant capacity of water soluble extracts from Parmigiano-Reggiano cheese. Int. J. Food Sci.
 471 Nutr. 64, 953-958.
- 472 Castillo, M., Martín-Orúe, S.M., Manzanilla, E.G., Badiola, I., Martín, M., Gasa, J., 2006. Quantification of
- total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. Vet. Microbiol.
- **474** 114, 165-70.
- 475 Condezo-Hoyos, L., Noratto, G., 2016. Aged American cheddar cheese as source of protein derived
- 476 compounds that modulate obese mice fecal bacteria and colon inflammation *in vitro*. Symbiosis. 4(5), 1-

477 10.

- 478 De Noni, I., Cattaneo, S., 2010. Occurrence of b-casomorphins 5 and 7 in commercial dairy products and in
 479 their digests following in vitro simulated gastro-intestinal digestion. Food Chem. 119, 560–566.
- 480 Duncan, S.H., Barcenilla, A., Stewart, C.S., Pryde, S.E., Flint, H.J., 2002. Acetate utilization and butyryl
- 481 coenzyme A (CoA):acetate-CoA transferase in butyrate-producing bacteria from the human large
- 482 intestine. Appl. Environ. Microbiol. 68, 5186-5190.
- 483 European Commission, 2009. Amendment application to Council Regulation (EC) No 510/2006 pursuant to
- 484 Article 9 'PARMIGIANO REGGIANO' EC No: IT-PDO-0317-0016-26.7.2007. Official J. Europ.
 485 Union. C87/06, 14-19.
- 486 European Commission, 2011. Commission Regulation (EU) No 794/2011 approving amendments to the
- 487 specification for a name entered in the register of protected designations of origin and protected
- 488 geographical indications (Parmigiano Reggiano (PDO)). Official J. Europ. Union. L204, 19-20.
- Fox, P.F., McSweeney, P.L.H., 1998. Dairy Chemistry and Biochemistry. Blackie Academic and
 Professional Publishers, London.
- 491 Gatti, M., Bottari, B., Lazzi, C., Neviani, E., Mucchetti, G., 2014. Invited review: Microbial evolution in
- raw-milk, long-ripened cheeses produced using undefined natural whey starters. J Dairy Sci. 97, 573-591.
- Gupta, A., Mann, B., Kumar, R., Sangwan, R., 2009. Antioxidant activity of Cheddar cheeses at different
 stages of ripening. Int. J. Dairy Tech. 62, 339-347.
- 495 Hernàndez-Ledesma, B., Martìnez-Maqueda, D., Miralles, B., Amigo, L., Gòmez-Ruiz, J.Á. 2012. *Peptides*.
- In: Nollet, L.M.L., Toldrà, F. (Eds.) Food Analysis by HPLC, Third Edition. CRC Press, Boca Raton,
- 497 USA, pp. 69-95.
- Ibrahim, S. A. Bezkorovainy, A., 1994. Growth-promoting factors for *Bifidobacterium longum*. J. Food. Sc.
 59, 189–191.
- Kieronczyk, A., Skeie, S., Olsen K., Langsrud, T., 2001. Metabolism of amino acids by resting cells of nonstarter lactobacilli in relation to flavour development in cheese. Intern. Dairy J. 11, 217-224.

- Kim, S. B., Lim, J. W., 2004. Calcium-binding peptides derived from tryptic hydrolysates of cheese whey
 protein. Asian-Aust. J. Anim. Sci. 17, 1459-1464.
- Korhonen, H., 2009. Milk-derived bioactive peptides: from science to applications. J. Funct. Foods 1, 177187.
- 506 Liepke, C., Adermann, K., Raida, M., Mägert, H. J., Forssmann, W. G., Zucht, H. D., 2002. Human milk
- 507 provides peptides highly stimulating the growth of Bifidobacteria. Eur. J. Biochem. 269, 712-718.
- Massart, D.L., Vandenginste, B.G.M., Buydens, I.M.C., De Jong S., Lewi P.J., Smeyers-Verbeke J., 1997.
- 509 Handbook of Chemometrics and Qualimetrics: Part A. Elsevier, Amsterdam, pp. 519-556.
- 510 McSweeney, P.L.H., 2004. Biochemistry of cheese ripening. Int. J. Dairy Tech. 57, 127-144.
- Meli, F., Lazzi, C., Neviani, E., Gatti, M., 2013. Effect of protein hydrolizates on growth kinetics and
 aminopeptidase activities of some *Bifidobacterium* species. Anaerobe 22, 130-133.
- Meli, F., Lazzi, C., Neviani, E., Gatti, M., 2014. Effect of protein hydrolysates on growth kinetics and
 aminopeptidase activities of *Lactobacillus*. Curr. Microbiol. 68, 82-87.
- 515 Minekus, M., Alminger, M., Alvito, P., Ballance, S., Bohn, T., Bourlieu, C., Carrière, F., Boutrou, Corredig,
- 516 M., Dupont, D., Dufour, C., Egger, L., Golding, M., Karakaya, S., Kirkhus, B., Le Feunteun, S., Lesmes,
- 517 U., Macierzanka, A., Mackie, A., Marze, S., McClements, D.J., M'enard, O., Recio, I., Santos, C.N.,
- 518 Singh, R.P., Vegarud, G.E., Wickham, M.S.J., Weitschies, W., Brodkorb, A., 2014. A standardised static
- *in vitro* digestion method suitable for food an international consensus. Food Funct. 5, 1113–1124.
- 520 Nadkarni, M.A., Martin, F.E., Jacques, N.A., Hunter, N., 2002. Determination of bacterial load by real-time
- 521 PCR using a broad-range (universal) probe and primers set. Microbiology 148, 257-266.
- 522 Nguyen, D.D., Johnson, S.K., Busetti, F., Solah, V.A., 2015. Formation and Degradation of Beta-
- 523 casomorphins in Dairy Processing. Crit. Rev. Food Sci. Nutr. 55, 1955-1967.
- 524 Oda, H., Wakabayashi, H., Yamauchi, K., Sato, T., Xiao, J.Z., Abe, F., Iwatsuki, K. 2013. Isolation of a
- 525 bifidogenic peptide from the pepsin hydrolysate of bovine lactoferrin. Appl Environ Microbiol. 79, 1843-
- 526 1849.

- 527 Olivi, O., Balli, F., Crema, G., Squadrini, F., Taparelli, F., 1979. Changes induced by diet therapy with
- casein hydrolysate on plasmatic free amino acids in infants with viral enteritis. Minerva Pediatr. 31, 14591467.
- Pancaldi, M., Mariotti, I., Balli, F., 2008. Intestinal inflammation in nursing infants: different causes and a
 single treatment... but of protected origin. Acta Biomed. 79, 144-150.
- 532Pinto, G., Caira, S., Cuollo, M., Lilla, S., Chianese, L., Addeo, F., 2012. Bioactive casein phosphopeptides in
- *dairy products as nutraceuticals for functional foods*. In: Hurley, W.L. (Ed.), Milk Protein. Intech,
 Rijeka, pp. 3-44.
- Poch, M., Bezkorovainy, A., 1991. Bovine milk k-casein trypsin digest is a growth enhancer for the genus *Bifidobacterium*. J. Agric. Food Chem. 39, 73-77.
- 537 Quartieri, A., Simone, M., Gozzoli, C., Popovic, M., D'Auria, G., Amaretti, A., Raimondi, S., Rossi, M.,
- 538 2016. Comparison of culture-dependent and independent approaches to characterize fecal bifidobacteria539 and lactobacilli. Anaerobe 38, 130-137.
- Richardson, A.J., McKain, N., Wallace, R.J., 2013. Ammonia production by human faecal bacteria, and the
 enumeration, isolation and characterization of bacteria capable of growth on peptides and amino acids.
- 542 BMC Microbiol. 13, 6.
- 543 Rinttilä, T., Kassinen, A., Malinen, E., Krogius, L., Palva, A.J., 2004. Development of an extensive set of
- 544 16S rDNA-targeted primers for quantification of pathogenic and indigenous bacteria in faecal samples by
- real-time PCR. Appl. Microbiol. 97, 1166-1177.
- 546 Rizzello, C.G., Losito, I., Gobbetti, M., Carbonara, T., De Bari, M. D., Zambonin, P.G., 2005. Antibacterial
- activities of peptides from the water-soluble extracts of Italian cheese varieties. J. Dairy Sci. 88, 23482360.
- 549 Rossi, M., Amaretti, A., 2010. Probiotic properties of bifidobacteria. In: van Synderen, D., Mayo, B. (Eds.),
- 550 Bifidobacteria: Genomics and Molecular Aspects. Horizon Scientific Press, Rowan House, UK, pp. 97-
- 551 123.

- 552 Rossi, M., Martínez-Martínez, D., Amaretti, A., Ulrici, A., Raimondi, S., Moya, A., 2016. Mining
- 553 metagenomic whole genome sequences revealed subdominant but constant *Lactobacillus* population in
- the human gut microbiota. *Environ. Microbiol. Rep.* 8, 399-406.
- Sekirov, I., Russell, S.L., Antunes, L.C., Finlay, B.B., 2010. Gut microbiota in health and disease. Physiol.
 Rev. 90, 859-904.
- Sforza, S., Cavatorta, V., Galaverna, G., Dossena, A., Marchelli, R., 2009. Accumulation of non-proteolytic
 aminoacyl derivatives in Parmigiano-Reggiano cheese during ripening. Int. Dairy J. 19, 582-587.
- 559 Sforza, S., Cavatorta, V., Lambertini, F., Galaverna, G., Marchelli, R., 2012. Cheese peptidomics: a detailed
- study on the evolution of the oligopeptide fraction in Parmigiano-Reggiano cheese from curd to 24
- 561 months of aging. J. Dairy Sci. 95, 3514-3526.
- 562 Sgarbi E., Bottari B., Gatti M., Neviani, E., 2014. Investigation of the ability of dairy nonstarter lactic acid
- bacteria to grow using cell lysates of other lactic acid bacteria as the exclusive source of nutrients. Int. J.
 Dairy Technol. 67, 342–347.
- 565 Simone, M., Gozzoli, C., Quartieri, A., Mazzola, G., Di Gioia, D., Amaretti, A., Raimondi, S., Rossi, M.,
- 566 2014. The probiotic *Bifidobacterium breve* B632 inhibited the growth of *Enterobacteriaceae* within
- colicky infant microbiota cultures. Biomed. Res. Int. 2014, 301053.
- Tidona, F., Criscione, A., Guastella, A.M., Zuccaro, A., Bordonaro, S., Marletta, D., 2009. Bioactive
 peptides in dairy products. Ital. J. Anim. Sci. 8, 315-340.
- 570 Visser, S., 1993. Proteolytic enzymes and their relation to cheese ripening and flavor: an overview. J. Dairy
 571 Sci. 76, 329-350.
- 572 Walker, A.W., Duncan, S.H., McWilliam Leitch, E.C., Child, M.W., Flint, H.J., 2005. pH and peptide supply
- 573 can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities
- from the human colon. Appl. Environ. Microbiol. 71, 3692-3700.
- Walter, J., 2008. Ecological role of lactobacilli in the gastrointestinal tract: implications for fundamental and
 biomedical research. Appl. Environ. Microbiol. 74, 4985-4996.

577 Wold, S., Esbensen, K., Geladi, P., 1987. Principal component analysis. Chemom. Intell. Lab. Syst. 2, 37-52.

578 Figure legends

- 579 Figure 1. Overlaid Full Scan chromatograms obtained for the waters soluble extracts (Panel A) and the
- 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
- 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 (OD₆₀₀) and
- impedance (E%), respectively. Values are means, n = 3, SD < 0.1. The box and whiskers plots (**Panel C**)
- report, for each species or group of species/subspecies, the increase in the different media. Boxes indicate the
- median and 25^{th} and 75^{th} percentiles; whiskers indicate the minimum and the maximum.
- 588 Figure 3. Peptide consumption by *Bifidobacterium* and *Lactobacillus* strains cultured for 24 h in BM
- 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
- (deepest green) to the highest (deepest red) consumption. Values are means, n = 3, SD always < 7.
- Figure 4. PCA model of peptide consumption by *Bifidobacterium* and *Lactobacillus* strains: PC1-PC2 score
 plot (a) and loading plot (b).
- **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,
- dashed line; relative amount of bifidobacteria in the microbiota, solid line. Values are means \pm 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 2 Revised Click here to download high resolution image



Figure 3 Click here to download high resolution image





Figure 5 Click here to download high resolution image





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	а	b	с	Х	У	Z	PROTEIN
12.3	278	EM					150.0 (1)	262 (2)	γ-Glu-Met
12.3	246	EV					118.1 (1)	230 (2)	γ-Glu-Val
18.3	310	EY		129.8 (1)	146.9 (1)		182.1 (1)	165 (1)	γ-Glu-Tyr
19.8	226	EP	83.8 (1); 181.1 (2)	209.1 (2)			115.9 (1)		pyro-Pro
20.8	242	EL	83.8 (1); 197.1 (2)	225.2 (2)	128.9 (1)		131.9 (1)		pyro-Leu
23.5	269	NH	224.1 (2)	252.1 (2)			156.0 (1)		
23.7	407	EEM					149.9 (1); 279.1 (2	2) 132.9 (1)	
24.1	230	IV	86 (1)				118.2 (1)		
24.6	375	EEV		130 (1); 358.5 (3)	147.8 (1)		247.1 (2); 118.0 (1) 230 (2)	
24.9	215	Y	170 (1)	198.1 (1)					
27.23	228	EV	83.7 (1); 183.1 (2)	211.1 (2)			118 (1)		pyro-Val
27.78	260	EI					131.9 (1)		γ-Glu-ile
28.26	228	LP	183.1 (2)	211.2 (2)			229.2 (2); 115.7 (1	.)	
29.0	260	EL		129.9 (1)			132.0 (1)	244.1 (2)	γ-Glu-leu
30.0	262	IM	86.2 (1); 216.9 (2)				149.9 (1)		
30.1	1155	S <u>SS</u> EESITR							
30.2	221	N-lactosyl-Met	176 (1)	204 (1)					
30.4	253	N-lactosyl-Tyr	208.1 (1)	236.1 (2)					
31.3	294	EF		129.9 (1)			166 (1)	278 (2)	γ-Glu-Phe
32.4	189	N-lactosyl-Val	144 (1)	172 (1)					
32.7	389	EEI		130 (1)			261.2 (2); 132.1 (1) 244.3 (2)	
33.9	1348	L <u>SSS</u> EESITR	86 (1)				605.2 (5); 476.3 (4): 276 1 (2)		β-casein
34.2	571	EIVPN					230.1 (2); 329.1 (3	5)	α_{s_1} -casein
34.6	242	EI	83.7 (1); 197.1 (2)	225.2 (2)	128.8 (1)		131.8 (1)	,	pyro-Ile
35.5	333	EW		129.8 (1)			205.3 (1)	317.3 (2); 188.1 (1)
36.1	1034	<u>SS</u> EEIVPN	777.2 (6)	706.1 (5); 805.1 (6))		571 (5); 329.2 (3); 230 (2); 133.7 (1)		α_{S1} -casein
36.4	242	EL	83.7 (1); 197.1 (2)	225.2 (2)	128.8 (1)		131.8 (1)		pyro-Leu
36.6	954	SSEEIVPN		725.2 (6)			329 (3); 230.6 (2)		α_{s_1} -casein
36.8	244	ĪL	86.0 (1); 199.0 (2)	227.1 (2)	131.6 (1)		132 (1)		51
37.8	278	FI	119.7 (1); 233.2 (2)	261.1 (2)			132 (1); 279.2 (2)		
38.9	278	IF	85.8 (1)				166 (1)		
39.1	1702	PSGAWYYVPLGTQYT		711.9 (13, 2+)			760.9 (13, 2+); 53 (14, 3+)	6	α_{S2} -casein
40.1	203	N-lactosyl-Ile/Leu							
40.2	1999	E <u>SLSSS</u> EESITRINK	101.9 (1)	628.7 (9, 2+)			530.8 (4); 416.9 (7 2+); 796.4 (12, 2+ 530.8 (12, 3+)	7, 357 (3); 563.4 (13,);3+)	β-casein

40.4	1870	QFLPYPYYAKPAAVRS		129.1 (1)			262.4 (2)		α_{S1} -casein
40.5	278	FI/L	120.0 (1); 233.4 (2)				131.8 (1)		
40.6	2051	KHYOKALNEINOFYOK					585.4 (4)		α_{s_2} -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	POLEIVPNSAEERLHSMKEG	912.9 (16, 2+)				723.3 (19, 3+)	524 (14, 3+)	α_{s_1} -casein
45	1938	TOTPVVVPPFLOPEVMGV		230.2 (2)		571.8 (10, 2+)	855.6 (16, 2+)		β-casein
45.9	2877	SLSQSKVLPVPQKAVPYPQRDMPIQA	201.2 (6, 3+)	201.2 (2); 100.7 (2, 2+); 520.1 (10, 2+) 817.6 (22, 3+); 887.8 (24, 3+); 930.8 (25, 3+)	, 218.3 (2); 520.1 ;(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+)		β-casein
47.1	3579	EELNVPGEIVE <u>S</u> L <u>SSS</u> EESITRINKKIEK	955.1 (9)	486.8 (4); 244.1 (4, 2+); 829.8 (14, 2+) 404.8 (11, 3+)	, 146.9 (1) ;	754 (13, 2+)	146.9 (1); 486.8 (12, 3+); 791.1 (18))	β-casein
49.2	2763	RPKHPIKHQGLPQEVLNENLLRF	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+)	445.5 (7, 2+); 501 (8, 2+)	α_{S1} -casein
49.4	3451	DAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGS		322.2 (3)	650.8 (17)		905.6 (17)	650.8 (19)	α_{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+) 761.5 (13, 2+)	816.9 (14, 2+); ;599.4 (15, 3+)	
50.5	5948	QEVLNENLLRFFVAPFPEVFGKEKVNELSKDIG SESTEDQAMEDIKQMEAES	946.9 (16, 2+); 1336.0 (35)	5128.9 (1); 679.3 (11, 2+)		595 (15, 3+)	656.2 (11, 2+)	226.2 (31, 3+)	
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+)	,	α_{S1} -casein
52.4	7182	KEAMAPKHKEMPFPKYPVEPFTESQSLTLTDV ENLHLPLPLLQSWMHQPHQPLPPTVMFPPQ	451.3 (12, 3+); 500.6 (13, 3+)	129.2 (1); 812.4 (14, 2+)		135.8 (2, 2+)	451.3 (12, 3+)		β-casein
52.5	3193	KEKVNEL <u>S</u> KDIG <u>S</u> E <u>S</u> TEDQAMEDIKQ		129.1 (1); 129.1 (2, 2+); 129.1 (3, 3+); 810.4 (20, 3+)	,	911.8 (22, 3+)		129.1 (2, 2+)	α_{S1} -casein
52.7	7054	EAMAPKHKEMPFPKYPVEPFTESQSLTLTDVE NLHLPLPLLQSWMHQPHQPLPPTVMFPPQ	810.5 (21, 3+)		259.8 (5); 633.4 (16, 3+)		917.0 (8); 677.2 (12, 2+); 451.4 (12, 3+)		β-casein
54.2	5486	<u>S</u> TEDQAMEDIKQMEAE <u>S</u> IS <u>S S</u> EEIVPNSVEQKH 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+)		α_{S1} -casein
55.2	4237	HIQKEDVPSERYLGYLEQLLRLKKYKVPQLEI VPN	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+)	213.1 (2); 213.1 (4); 802.5 (20, 3+)	α_{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);		

57.4 12262 KHPIKHQGLPQEVLNENLLRFFVAPFPEVFGK EKVNELSKDIGSESTEDQAMEDIKQMEAESISS SEELVPNSVEQKHIQKEDVPSERYLGYLEQLLR 129.2 (1) 1343.9 (33, 3+) a _{S1} -ca 58.0 3602 KEDVPSERYLGYLEQLLRLKKYKVPQLEIVN IKKYK 187.1 (2); 1082 (27, 3) 230.1 (2) 326 (8, 3+) 565.3 (14, 3+) 1082 (18, 2+) 60.1 1881 YQQPVLGPVRGPFPIIV 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+) 164.8 (29, 2+); 60.1 1882 LEELNVPGEIVESLSSS-p 85.8 (1) 1094.7 (10) 616.1 (5) 181.1 (3, 2+) 164.8 (29, 2+); 61.4 (3); 149.3 (14, 2+) 164.8 (29, 2+); 61.4 (2); 1461.8 (14); 714.7 (13, 314); 1649.8 (29, 2+); 62.4 (17, 3+); 714.7 (164.9 (12), 453.1 (9, 365.3 (7, 2+)); 620.4 (6); 756.1 (14, 2+) 1649.8 (29, 2+); 62.4 (17, 3+); 714.7 (165.7 (29, 3+); 106.7 (29, 3+); 106.7 (29, 3+); 104.2 (11, 2+) 104.2 (11, 3+); 62.4 (17, 3+); 714.7 (165.7 (20, 151.3 (40, 3+)) 3+)								909.5 (8); 1162.6 (29, 3+)		
58.0 3602 KEDVPSERYLGYLEQLLRLKKYKVPQLEIVPN 3+) 187.1 (2); 1082 (27, 230.1 (2) 3+) 326 (8, 3+) 565.3 (14, 3+) 1082 (18, 2+) 60.1 1881 YQQPVLGPVRGPFPIIV 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+) 60.1 1882 LEELNVPGEIVESLSSS-p 85.8 (1) 1094.7 (10) 616.1 (5) 181.1 (3, 2+) 61.8 8715 MGVSKVKEAMAPKHKEMPFPKYPVEPFTESQ SLTLTDVENLHLPLPLLQSWMHQPHQPLPPTV 2+); 1100.3 (20, 2+); 1658.6 (29, 2+); (21, 3+) 365.3 (7, 2+); (22, 4); 774.7 620.4 (6); 756.1 (14, 2+) 762.2 (7); 774.7 1649.8 (29, 2+); (14, 2+); 1391.2 756.1 (21, 3+); (25, 2+); 762.2 (21, 1100.3 (29, 3+); (25, 2+); 762.2 (21, 1100.3 (29, 3+); (21, 3+) 1006.7 (29, 3+); (21, 3+) 310.6 (40, 3+) 3+) 68.3 4023 SLVYPFPGPIPNSLPQNIPPLTQTPVVPPFLQP EVM 227.2 (6, 3+) 707.4 (6); 1071.7 (10); 1936 (18); 968.3 (18); 1938.9 326.9 (2, 3+) (9) 1058.6 (19, 2+) (9) 475.3 (4); 1057.4 908.7 (27, 3+) (9) (9)	57.4	12262	KHPIKHQGLPQEVLNENLLRFFVAPFPEVFGK EKVNELSKDIG <u>S</u> ESTEDQAMEDIKQMEAESI <u>SS</u> <u>S</u> EEIVPNSVEQKHIQKEDVPSERYLGYLEQLLR LKKYK		129.2 (1)			1343.9 (33, 3+)		α_{S1} -casein
60.1 1881 YQQPVLGPVRGPFPIIV 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) 292.2 (2); 421.2 (3); 616.1 (5) 60.1 1882 LEELNVPGEIVESLSSS-p 85.8 (1) 1094.7 (10) 616.1 (5) 181.1 (3, 2+) 61.8 8715 MGVSKVKEAMAPKHKEMPFPKYPVEPFTESQ 1106.7 (11); 453.1 (9, 365.3 (7, 2+); 620.4 (6); 756.1 620.4 (6); 756.1 762.2 (7); 774.7 1649.8 (29, 2+); β-case 61.8 8715 MGVSKVKEAMAPKHKEMPFPKYPVEPFTESQ 1106.7 (11); 453.1 (9, 365.3 (7, 2+); 620.4 (17, 3+); 714.7 453.1 (13, 3+); (25, 2+); 762.2 (21, 1100.3 (29, 3+); 100.3 (20, 2+); 1658.6 (29, 2+); (21, 3+); (25, 2+); 762.2 (21, 1100.3 (29, 3+); 100.3 (29, 3+); 106.7 (29, 3+); 1106.7 (29, 3+); 1106.7 (29, 3+); 1106.7 (29, 3+); 1106.7 (29, 3+); 1106.7 (29, 3+); 1142.5 (30, 3+) 3+) 68.3 4023 SLVYPFPGPIPNSLPQNIPPLTQTPVVPPFLQP 227.2 (6, 3+) 707.4 (6); 1071.7 (0); 1936 (18); 968.3 (18); 1483.9 (28, 2+) 475.3 (4); 1057.4 908.7 (27, 3+) (9) 68.3 4023 SLVYPFPGPIPNSLPQNIPPLTQTPVVPPFLQP 227.2 (6, 3+) 707.4 (6); 1071.7 (0); 1936 (18); 968.3 (18); 1483.9 (28, 2+) 475.3 (4); 1057.4 908.7 (27, 3+) (9) 908.7 (27, 3+) (9) 908.7 (27, 3+) (9) 908.7 (27, 3+) (9) 908.7 (27, 3+) (9) 908.7 (27, 3+) (9) 908.7 (27, 3+) (9) 908.7 (27, 3+) (9)	58.0	3602	KEDVPSERYLGYLEQLLRLKKYKVPQLEIVPN	187.1 (2); 1082 (27, 3+)	230.1 (2)	326 (8, 3+)	565.3 (14, 3+)		1082 (18, 2+)	
60.1 1882 LEELNVPGEIVESLSSS-p 85.8 (1) 1094.7 (10) 616.1 (5) 181.1 (3, 2+) 61.8 8715 MGVSKVKEAMAPKHKEMPFPKYPVEPFTESQ 1106.7 (11); 453.1 (9, 365.3 (7, 2+); SLTLTDVENLHLPLPLLQSWMHQPHQPLPPTV 2+); 1100.3 (20, 2+); 620.4 (17, 3+); 774.7 620.4 (6); 756.1 762.2 (7); 774.7 1649.8 (29, 2+); (14, 2+); 1391.2 756.1 (21, 3+); (25, 2+); 762.2 (21, 1100.3 (29, 3+); (21, 3+) 620.4 (17, 3+); 774.7 453.1 (13, 3+); (21, 3+) (106.7 (29, 3+); 1801 (48, 3+) 3+); 1106.7 (29, 3+) 3+); 1106.7 (29, 3+) 3+); (3+) 68.3 4023 SLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQP EVM 227.2 (6, 3+) 707.4 (6); 1071.7 (10); 1936 (18); 968.3 (18); 1483.9 (28,2+) 1058.6 (19, 2+) (10); 1936 (18); 968.3 (18); 1483.9 (28,2+) 1058.6 (19, 2+) (9) 475.3 (4); 1057.4 908.7 (27, 3+) (9)	60.1	1881	YQQPVLGPVRGPFPIIV	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+)		
61.8 8715 MGVSKVKEAMAPKHKEMPFPKYPVEPFTESQ 1106.7 (11); 453.1 (9, 365.3 (7, 2+); SLTLTDVENLHLPLPLLQSWMHQPHQPLPPTV 620.4 (6); 756.1 762.2 (7); 774.7 1649.8 (29, 2+); (14, 2+); 1391.2 756.1 (21, 3+); (25, 2+); 762.2 (21, 1100.3 (29, 3+); (25, 2+); 762.2 (21, 1100.3 (29, 3+); (21, 3+) 620.4 (17, 3+); 774.7 453.1 (13, 3+); (21, 3+) (106.7 (29, 3+); (21, 3+) 1106.7 (29, 3+); (21, 3+) 707.4 (6); 1071.7 326.9 (2, 3+) 3+); 1102.7 (29, 3+); (3+) 3+); 68.3 4023 SLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQP EVM 227.2 (6, 3+) 707.4 (6); 1071.7 326.9 (2, 3+) 1058.6 (19, 2+) 475.3 (4); 1057.4 908.7 (27, 3+) (10); 1936 (18); 968.3 (18); 1483.9 (28,2+) 908.3 (18); 1483.9 (28,2+) 908.3 (18); 1483.9 (28,2+) 908.7 (27, 3+) 908.7 (27, 3+)	60.1	1882	LEELNVPGEIVESLSSS-p	85.8 (1)	1094.7 (10)	616.1 (5)		181.1 (3, 2+)		
68.3 4023 SLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQP 227.2 (6, 3+) 707.4 (6); 1071.7 326.9 (2, 3+) 1058.6 (19, 2+) 475.3 (4); 1057.4 908.7 (27, 3+) EVM (10); 1936 (18); (9) (28,2+) (28,2+) (28,2+) (21, 22, 23, 23, 23, 23, 23, 23, 23, 23, 23	61.8	8715	MGVSKVKEAMAPKHKEMPFPKYPVEPFTESQ SLTLTDVENLHLPLPLLQSWMHQPHQPLPPTV MFPPQSVLSLSQSK	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+); 1100.3 (29, 3+); 1513.6 (40, 3+)	β-casein
	68.3	4023	SLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQP EVM	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).

#	$\mathbf{r}_{\mathrm{T}(\mathrm{min})}$	n) MW	SEQUENCE	Precurso Ion	r a	b	c	Р	У	Z	PROTEIN
P1	9.9	614	LHSMK	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	EGIHAQQK	455		130.1 (1); 187.2 (2)			474.3 (4); 611.4 (5)		α_{s1} -casein
P3	17.8	715	n. d.								
P4	21.7	230	IV	231	86.1 (1); 185.2 (2)						
P5	23	548	YAKPA	549		363.1 (3); 531.3 (5)			315.2 (3)		к-casein
P6	25.7	781	EGIHAQQ	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_{s1} -casein
P7	27.6	830	EDVPSER	831					175.2 (1); 587.3 (5)	471.2 (4)	α_{s1} -casein
P8	27.6	260	EL	261		130.0 (1)			132.8 (1)	244.1 (2)	
P9	27.7	230	VI	231	71.9 (1); 185.2 (2)				132 (1)		
P10	27.8	351	YIG	352		277.2 (2)			189.1 (2)		
P11	27.8	526	EGIHA	525		187.0 (2); 437.2 (4); 508.3 (5)			227.2 (2); 340.4 (3)		α_{s1} -casein
P12	27.9	388	QEL	389	101.9 (1)	129.0 (1); 258.2 (2)			132.0 (1)		α_{s1} -casein
P13	28.4	1336	n. d.								
P14	29.7	1180	HIQKEDVPSE	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. (10, 2+)	1κ-casein
P15	30	663	TESQSL	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	AMEDIK	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_{s1} -casein
P17	30.7	679	DAYPSGA	678		115.9 (1); 187.0 (2); 350.1 (3)			331.3 (4); 234.2 (3)		α_{s1} -casein
P18	30.8	276	AW	275	230.2 (2)	258.2 (2)			205.2 (1)	188.2 (1)	
P19	31.1	633	YKVPQ	634	292.1 (2);				343.3 (3); 244.2 (2); 147.2 (1)		α_{s1} -casein
P20	31.4	294	YI / IY / YL / LY	295	136.0 (1); 249.2 (2)				132 (1)	278.2 (2)	
P21	32.2	330	PYPQR	659		261.1 (2)			563.3 (4); 400.2 (3); 175.2 (1)		β-casein
P22	32.4	420	QSW	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	HKEMPFPK	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	TLTDVE	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	VLPVPQK	780					372.3 (3); 568.4 (5)		β-casein
P26	34.7	675	AVPYPQ	674		431.2 (4);			244.1 (2); 504.2 (4)		β-casein
P27	36.7	512	IHPF	513					166.0 (1); 263.0 (2)		β-casein
P28	37	501	LEQL	502		243.1 (2); 371.1 (3); 484.3 (4)			260.2 (2)	243.1 (2)	α_{s2} -casein

P29	37.7	812	NAVPITPT	811		285.1 (3); 495.3 (5); 596.4 (6);		627.5 (6); 528.3 (5); 318.2 (3); 217.1 (2);		α_{s2} -casein
P30	38.7	558	ALNEI	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)	α_{s2} -casein
P31	39.1	674	DMPIQA	673		247.1 (2); 344.2 (3); 457.2 (4); 585.3 (5)		428.3 (4); 218.2 (2);	411.2 (4);	β-casein
P32	39.4	651	VLPVPQ	652		310.2 (3); 409.3 (4);		244.1 (2)		β-casein
P33	40.7	1512	MHQPHQPLPPTVM	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)		β-casein
P34	41	562	TTMPL	561				360.2 (3); 229.3 (2)		α_{s1} -casein
P35	42.7	587	PVEPF	588		326.2 (3);		1661. (1); 263.3 (2); 392.2 (3);		β-casein
P36	43.7	754	SDIPNPI	755		527.3 (5)		553.5 (5); 440.3 (4); 229.2 (2)	738.6 (7); 212.1 (2)	α_{s1} -casein
P37	43.7	645	EPMIGV	644		358.1 (3); 471.2 (4); 528.2 (5); 627.3 (5)		419.2 (4); 288.2 (3); 175.2 (2);		α_{s1} -casein
P38	44.9	504	YIPI	505	136.1 (1); 249.1 (2)			131.9 (1); 229.4 (2);		κ-casein
P39	45.2	729	Q/KNDEPK/Q	730	459.2 (4)	129.1 (1)		147.1 (1); 244.3 (2); 488.4 (4)		
P40	45.6	685	RGPFPI	686		157.1 (1);		229.2 (2)	669.2 (6)	β-casein
P41	45.7	1139	IA <u>S</u> GEPTSTPT	571		635.5 (5)		217.1 (2); 317.8 (3);		α_{s1} -casein
P42	46	721	GYLEQL	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)	α_{s1} -casein
P43	46.7	750	YPVEPF	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)		β-casein
P44	47.6	1462	EEYSIGS <u>SS</u> EES	732				698.6 (5)		α_{s2} -casein
P45	48.1	653	YYVPL	654		426.3 (3)		229.2(2); 328.3 (3)		α_{s1} -casein
P46	48.6	757	VAPFPEV	758	387.3 (4)	268.2 (3); 415.2 (5);		344.2 (3); 491.3 (4); 588.3 (5);		α_{s1} -casein
P47	48.9	820	VLSRYPS	821	272.3 (3)			609.2 (5); 522.2 (4)		α_{s1} -casein
P48	49	530	GPFPI	520		155 (2); 302.2 (3)		376.3 (3); 229.3 (2)		β-casein
P49	50	688	LHLPLP	689		251.2 (2); 461.3 (4);		229.2 (2); 439.3 (4);		β-casein
P50	51	1100	VYPFPGPIPN	1099	730.4 (7)	871.5 (8)		230.3 (2); 440.2 (4); 594.4 (6); 838.5 (8)	1083.6 (10)	β-casein
P51	52.3	1372	TDAPSFSDIPNPI	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)	α_{s1} -casein
P52	52.4	1267	INNQFLPYPY	1268		730.5 (6); 990.6 (8)		279.3 (2); 539.3 (4); 652.4 (5)		κ-casein
P53	52.8	829	YQGPIVLNPWDQVK	1656	136.1 (1)	292.2 (2); 349.1 (3); 18 658.3 (6)	81.2 (1)	772.5 (6); 1000.6 (8); 1309.9 (11); 1366.3 (12);		α_{s2} -casein
P54	53.7	2429	HIQKEDVPSERYLGYLEQLL	1216		741.5 (12); 827.1 (14)			1017.9 (17)	α_{s1} -casein
P55	54.3	1479	EMPFPKYPVEPF	740		1218.8 (10)		166.2 (1); 263.3 (2); 976.7 (8); 611 (10, 2+)	958.8 (8)	β-casein
P56	54.4	888	VYPFPGPI	889				383.3 (4); 627.3 (7)		β-casein
P57	54.6	904	FVAPFPEV	905		415.3 (4); 562.3 (5); 659.4 (6)		344.1 (3); 491.3 (4); 588.3 (5); 659.4 (6)		α_{s1} -casein
P58	54.8	1299	SLVYPFPGPIPN	1300		804.3 (7); 1071.6 (10)		594.4 (6); 838.5 (8); 1001.6 (9)		β-casein
P59	55.1	1236	FVAPFPEVFGK	619	119.9 (1); 219.3 (2); 316.1 (6, 2+)	247.2 (2); 318.2 (3) 67	76.5 (6)	676.5 (6); 823.4 (7); 920.5 (8); 991.5 (9); 412.3 (7, 2+); 460.8 (8, 2+); 496.4 (9, 2+)		α_{s1} -casein

P60	55.1	1248	PVLGPVRGPFPI	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+)		β-casein
P61	55.2	753	QEPVLGPVRGPFPI	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+)	β-casein
P62	55.7	643	GPFPII	642		155.1 (2); 302.3 (3); 399.4 (4); 512.3 (6)			489.4 (4); 342.3 (3); 245.1 (2);		β-casein
P63	56.6	1220	PVVVPPFLQPE	1221		296.5 (3); 395.3 (4); 492.4 (5); 736.6 (7); 849.7 (8); 977.7 (9)			827.6 (7)		β-casein
P64	57.5	741	GPFPIIV	742		625.4 (6); 512.3 (5);			441.3 (4);		β-casein
P65	60.1	1882	LEELNVPGEIVESLSSS-p		85.8 (1)	1094.7 (10)	616.1 (5)		181.1 (3, 2+)		
P66	60.6	1360	TDVENLHLPLPL	681		217.2 (2); 922.5 (8); 1132.7 (10)			229.3 (2)		β-casein
P67	61.3	1258	DVENLHLPLPL	630	187.2 (2)	821.5 (7); 1031.7 (9)			229.3 (2)		β-casein
P68	61.7	788	LLDAQSAPLRVYVE	1573	85.8 (1)				1347 (12)		β-lactoglobulin
P69	61.8	2511	SLPQNIPPLTQTPVVVPPFLQPE	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+)	β-casein
P70	63.4	2610	SLPQNIPPLTQTPVVVPPFLQPEV	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)		β-casein
P71	63.5	1700	n. d.								
P72	63.9	1774	n. d.								



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