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

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Abstract

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, some of which have potential healthy bioactive properties. This study aimed to: i) determine the peptide profile of PR cheese subjected to simulated gastrointestinal transit; ii) evaluate *in vitro* whether the peptides could support growth of beneficial microbial groups of the gut microbiota. PR samples were subjected to *in vitro* digestion, simulating oral, gastric, and duodenal transit. Liquid chromatography coupled with tandem mass spectrometry revealed that digestion caused the disappearance of the serum proteins and most of the 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), and to bioreactor batch cultures of human gut microbiota. Most of bifidobacteria and lactobacilli grew more abundantly on PR digests than on the control peptone, and exhibited strain- or species- specific peptide preferences, as evidenced by principal component analysis. Bifidobacteria generally consumed a greater amount of peptides than lactobacilli, in terms of both the mean peptide consumption and the number of peptides consumed. For bifidobacteria, peptide preferences were very diverse, but a core of 10 peptides with 4 or 5 residues were consumed by all the strains. Lactobacilli behaved more homogenously and consumed nearly only the same 6 peptides, mostly dipeptides. The peptide preferences of the different groups of 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 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 bifidobacteria. This study demonstrated that peptides derived from simulated gastrointestinal digestion of PR supported the growth of most lactobacilli and bifidobacteria.

Keywords

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

Abbreviations

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

1 Introduction

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 and natural whey starter and a Protected Designation of Origin, in compliance with the European norm currently in force (European Commission, 2009, 2011). The curd is heated at 55°C to select thermophilic 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 fractions, the ratio of fat to protein being around 0.94, depending on milk characteristics. Ripened PR is 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 mg) acids (Gatti et al., 2014). The overall mineral content of PR is 4.0 to 4.5%, with NaCl at approximately 1.5% (Gatti et al., 2014).

Long ripening times allow for cheese extensive proteolysis (Fox and McSweeney, 1998; McSweeney, 2004; Visser, 1993) due to the residual rennet activity and the enzymes of starter (SLAB) and non-starter (NSLAB) lactic acid bacteria. During ripening, proteolysis gives rise to the continuous evolution 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 improved digestibility, reduced allergenicity, and flavor development (Alessandri et al., 2012; Sforza et al., 2012). The potential biological activity of PR peptides has recently attracted particular interest (Korhonen et al., 2009; Tidona et al., 2009). *In vitro* studies revealed calcium binding properties (Kim et al., 2004; Pinto et al., 2012) and antioxidant (Bottesini et al. 2013; Gupta et al., 2009), antihypertensive (Bernabucci et al., 2014), antimicrobial activities (Benkerroum, 2010; Rizzello et al., 2005)..

The nutritional value of PR, coupled with the potential beneficial properties of bioactive peptides that could impact health, suggested its use as functional food in a dietary therapy for subjects with 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 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 high digestibility and the high amounts of short chain fatty acids, amino acids, and oligopeptides easily absorbed in the bowel likely support this beneficial effect. Furthermore, the hypoallergenicity, the absence of lactose, and the high sodium content that restores losses of salts and reduces the secretions of intestinal cells have been claimed as responsible of the positive outcome of this dietary therapy (Olivi et al., 1979; Pancaldi et al., 2008).

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 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 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 released with the gastric digestion of an aged cheese were demonstrated to modulate the composition of mice microbiota *in vitro*, improving the abundance of bifidobacteria (Condezo-Hoyos et al., 2016). On this basis it has been hypothesized that PR peptides could promote growth and/or activity of these beneficial microbes, 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 gastrointestinal digestion and, at least partially, reach the colon. The present study aimed to characterize the peptides derived from simulated gastrointestinal digestion of PR cheese, utilizing an *in vitro* digestion model that recently achieved an international consensus (Minekus et al., 2014). The resulting peptide mixtures were characterized by liquid chromatography coupled with mass spectrometry. In order to verify whether peptides specifically promoting the growth of beneficial bacteria and potential probiotics could be identified, the misxtures were tested as nitrogen source for bifidobacteria and lactobacilli in pure and fecal cultures.

2 Materials and Methods

2.1 Cheese water soluble extract

Samples of PR aged 16, 24, and 36 months (hereinafter referred to as PR16, PR24, and PR36, respectively) were provided by Consorzio del Formaggio Parmigiano Reggiano (Modena, Italy). Five grams of finely ground cheese were added with 45 mL of HCl 0.1 M and 1.25 mL of an aqueous solution of 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 proteins were removed by acid precipitation followed by centrifugation (45 min at 4°C at 3220 × g), according to Hernàndez-Ledesma et al. (2012). The supernatant was filtered through paper filter and extracted three times with diethyl ether to remove fats. Ether residues were removed with a rotavapor, and the residual solution was filtered subsequently through 5 µm and 0.45 µm filters.

2.1 Simulated gastrointestinal digestion of PR samples

All the chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) unless otherwise stated. The cheese was finely grounded and digested following the procedure, consisting in three main steps: 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 KH_2PO_4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2$, and 0.06 mM $(NH_4)_2CO_3$), 2.5 mL of 1500 U/mL amylase, 125 µL of 300 mM $CaCl_2$, and 4.875 mL of distilled water (ratio cheese:digestive fluid of 1:1, w:v). The sample was vortexed and incubated for 2 min at 37°C on a reciprocating shaker. Then, 37.5 mL of gastric buffer (6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.1 mM $MgCl_2$, and 0.5 mM $(NH_4)_2CO_3$), 8 mL of 25000 U/mL pepsin, 25 µL of 300 mM $CaCl_2$, 1 mL of 1 M HCl and 3.475 mL of water were added (final ratio cheese:digestive fluids 1:3, w:v). 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 KH_2PO_4, 85 mM $NaHCO_3$,

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 µL of 300 mM $CaCl_2$, 750 µL of 1 M NaOH and 6.55 mL of distilled water were added (final ratio cheese:digestive fluids 1:7, w:v). The pH was adjusted to 7 using 1 M NaOH. The sample was vortexed and incubated for 2 hours at 37°C on a reciprocating shaker. To stop the digestion the sample was heated at 95°C for 15 min, then cooled. Since the assessment of digestion is strongly affected by the analysis of the digested fraction, the samples were centrifuged for 45 min at 4°C at 3220 × *g* to remove insoluble proteins and undigested components (Minekus et al., 2014). The supernatant was filtered through 0.45 µm membranes. For chromatographic analysis, 196 µL of sample was supplemented with 4 µL of 1 mM Phe-Phe.

2.2 HPLC-ESI-MS/MS analysis.

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

Peptides were manually identified as follows. Molecular ions were recorded from the Full Scan 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 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 pattern of these sequences was determined (http://db.systemsbiology.net/proteomicsToolkit/FragIonServlet.html last accessed on September 2016) and compared with the MS/MS spectra. The peptide sequences were assigned on the basis of the matching fragments.

2.3 UPLC/ESI-MS analysis.

Absolute quantification of all the peptides identified was not possible due to the unavailability of all 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 performed using Phe-Phe as internal standard. The ratio between the chromatographic peak of the peptide and that of Phe-Phe did not yield absolute peptide concentration but allowed the comparison of the same 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 separated by a reverse phase column (Acquity UPLC BEH 300 C18, 1.7 µm, 2.1 × 150 mm equipped with a Acquity UPLC BEH C18 VanGuard Pre-column, 300Å, 1.7 µm, 2.1 × 5 mm, Waters) in an UPLC system coupled with ESI and MS (UPLC Acquity with a single quadrupole detector SQD, Waters). In order to replicate a chromatographic trace similar to HPLC, but with shorter run time, gradient elution with the same eluents was programmed as follows: 0-7 min, 100% A; 7-50 min, from 100% A to 50% A; 50-52.6 min, 50% 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, 100% A. The analysis parameters were: flow 0.2 mL/min; analysis time 72 min; column temperature 35°C;

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

2.4 Strains and culture conditions

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

2.5 Pure culture conditions

Utilization of PR digests was investigated in a basal medium (hereinafter referred to as BM) where PR16, PR24, PR36 digests or Bacto peptone (BD Difco) were utilized as nitrogen source, the latter as positive control. The medium contained lactose, 10 g/L; PR hydrolysates or Bacto peptone (BD Difco), 5 g/L; ammonium citrate, 2 g/L; yeast nitrogen base w/o amino acids and ammonium sulfate (BD Difco), 1.7 g/L; polysorbate 80, 1 g/L; sodium acetate trihydrate, 5 g/L; K_2HPO_4, 2 g/l; $MgSO_4 \cdot 7H_2O$, 0.1 g/l; $MnSO_4 \cdot H_2O$, 0.05 g/L; cysteine hydrochloride, 0.5 g/l; pH was corrected to 6.5. Bacteria inoculated (5% v/v) in BM based media were incubated 24 h at 37°C, and passaged at least three times before evaluation of growth and peptide consumption.

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
204 diluted 1:100 in Ringer's solution (Oxoid, Basingstoke, UK), then 0.1 ml were used to inoculate tubes
205 containing 6 mL of BM media. The impedance measurement was performed at 37°C. Bacteria growth was
206 measured as E%, where E-value is the impedance change revealed by the system at the electrode surface.
207 Variation in electrical conductivity, monitored during time, is proportional to the change in the number of
208 microorganisms (Bancalari et al., 2016). Maximum value of E% was recorded as index of maximum
209 microbial growth.
210
211 *2.6 Cultures of intestinal microbiota*
212 Batch cultures of fecal microbiota were performed in bioreactors containing 200 mL of FM medium,
213 developed from the media described by Walker et al. (2005) and Duncan et al. (2002), with some
214 modifications: beech wood xylan, 0.6 g/L; citrus pectin, 0.6 g/L; maize amylopectin, 0.6 g/L; larch wood
215 arabinogalactan, 0.6 g/L; potato starch, 3 g/L; fructans (Synergy1, Beneo, Mannheim, Germany), 3 g/L;
216 peptone, 5 g/L; ammonium citrate, 2 g/L; KH_2PO_4, 2 g/L; NaCl, 4.5 g/L; $MgSO_4 \cdot 7H_2O$ 0.5 g/L; $CaCl_2 \cdot$
217 $2H_2O$ 0.045 g/L; $FeSO_4 \cdot 7H_2O$, 0.005 g/L; hemin, 0.01 g/L; bile salts (Oxgall, BD Difco) 0.05 g/L,
218 resazurin, 0.6 mg/L; reducing solution, 40 ml/L; mineral solution, 2 ml/L; vitamin solution, 1.4 ml/L.
219 Reducing solution contained: cysteine hydrochloride, 12.5 g/L; $NaHCO_3$, 80 g/L. Mineral solution
220 contained: EDTA, 500 mg/L; $FeSO_4 \cdot 7H_2O$, 200 mg/L; $ZnSO_4 \cdot 7H_2O$, 10 mg/L; $MnCl_2 \cdot 7H_2O$, 3 mg/L;
221 H_3BO_3, 30 mg/L; $CoCl_2 \cdot 6H_2O$, 20 mg/L; $CuCl_2 \cdot 2H_2O$, 1 mg/L; $NiCl_2 \cdot 6H_2O$, 2 mg/L; $NaMoO_4 \cdot 2H_2O$, 3
222 mg/L. Vitamin solution contained: menadione, 1 g/L; biotin 2 g/L; calcium pantothenate 2 g/L; nicotinamide,
223 10 g/L; vitamin B12, 0.5 g/L; folic acid 0.5 g/L; thiamine, 4 g/L; PABA, 5 g/L. Reducing, mineral and
224 vitamin solutions were filter sterilized and added to the autoclaved medium. PR24 digest or Bacto peptone
225 were utilized as nitrogen source at the concentration of 5 g/L in parallel batches, carried out in duplicate. The
226 inoculum was prepared pooling equal amounts of fecal microbiota from three healthy adult subjects. The

feces were homogenized (5% w/v) with the medium in the anaerobic cabinet (Anaerobic System, Forma Scientific, Marietta, OH) under a 85% N_2, 10% CO_2, 5% H_2 atmosphere. 10 mL of the suspension were inoculated into bioreactors (Sixfors V3.01, Infors, Bottmingen, Switzerland) each containing 190 mL of fresh sterile medium, and the processes proceeded for 48 h (37°C, CO_2 atmosphere, pH 6.2, stirring 60 rpm). 5 mL samples were collected at 0, 6, 12, 24 h and stored at -20°C until analyzed.

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

2.7 Principal component analysis of peptide consumption

Principal component analysis (PCA) (Massard et al., 1997; Wold et al., 1987) was carried out to 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 the extracts. The experimental data were analyzed using PLS Toolbox software ver. 8.1.1 (Eigenvector Research Inc., Wenatchee, WA, USA) for the calculation of PCA models. A PCA model was calculated on a dataset with size {45, 63} including all the 45 samples (i.e. 15 strains × 3 ripening ages of PR) obtained by digestions carried out with bifidobacteria and lactobacilli, each one characterized by the consumption values of 63 peptides. Two further PCA models were calculated considering bifidobacteria and lactobacilli separately, i.e. on two separate datasets with size equal to {24, 63} and to {21, 63}, respectively. Before

252 calculating the PCA models, the variables were pre-processed using mean centering. The number of
253 significant PCs, i.e., the number of PCs bearing useful information, was selected based on the scree plot
254 reporting the percentage of variance explained by each PC vs. the PC number.
255
256 **3 Results**
257 *3.1 Effects of simulated digestion on the peptide profile of PR*
258 The peptide profile of PR16, PR24, and PR36 was characterized by HPLC/ESI-MS/MS before and
259 after the *in vitro* simulated gastrointestinal digestion (Figure 1A, 1B). Intact caseins are not taken into
260 account, since they were removed from both the water soluble extracts and from the digested samples.
261 Before the simulated digestion, a common peptide profile was detected in the water extracts of PR16, PR24,
262 and PR36 samples (Fig. 1A). 63 main peptides were identified, including some N-lactosyl amino acids, with
263 a length up to 103 residues and a molecular weight spread over a wide range (Supporting Information: Table
264 S1). 31 peptides had a MW < 500 Da, 2 had a MW of 500-1000 Da, 12 fell in the range 1000-2000 Da.
265 Longer peptides were also identified, 12 with MW of 2-5 kDa and at 6 with MW of 5-20 kDa. Moreover, the
266 two isoforms of β-lactoglobulin (A and B) were clearly detectable at 40.14 min.
267 After *in vitro* digestion, the mixtures of PR16, PR24, and PR36 still shared similar profile. The
268 digests lacked intact proteins, including β-lactoglobulins, and 61 out of the 63 peptides identified in water
269 soluble extracts were no longer detectable, being degraded into shorter ones (Fig. 1B). The sole exception
270 were FVAPFPEVFGK and the phosphopeptide LEELNVPGEIVESLSSS. The former was present in the
271 water soluble extract, especially of the PR36, and remained in similar amounts after digestion, whereas the
272 latter occurred in the digests in minor amount. N-lactosyl amino acids also resisted digestion, but their
273 chromatographic signal was negligible, compared with those of peptides.
274 As a whole, 72 main peptides were identified in the PR digests (Supporting Information: Table S2)
275 and, with the exception of P3, P13, P71, and P72, were assigned to specific sequences. Compared with the
276 undigested water extracts, molecular weight distribution of the peptides was shifted toward lower MW: 12

277 peptides had MW < 500 Da, 41 had a MW of 500-1000 Da, constituting the majority of peptides of the
278 digests, and 14 peptides had a MW of 1000-2000 Da. Only 3 peptides presented a MW > 2 kDa, none of
279 them exceeding 2700 Da. (Fig. 1B).
280
281 *3.2 Growth of bifidobacteria and lactobacilli on PR digests as nitrogen source*
282 Twenty seven *Bifidobacterium* strains belonging to the species *B. adolescentis*, *B. animalis* subsp.
283 *animalis*, *B. animalis* subsp. *lactis*, *B. bifidum*, *B. breve*, *B. longum* subsp. *infantis*, *B. longum* subsp. *longum*,
284 and *B. pseudocatenulatum*, and 30 *Lactobacillus* strains belonging to the species *L. casei*, *L. paracasei* and
285 *L. rhamnosus* were investigated for the ability to grow utilizing PR hydrolysates as nitrogen source. All the
286 strains grew both in the control medium and the PR-based media (Fig. 2), exhibiting a species specific
287 growth yield, with few exceptions. Within the phylogenetically related group of *B. adolescentis* and *B.*
288 *pseudocatenulatum*, the strains belonging to the former species gave similar yields in the diverse BM media,
289 while the strains belonging to the latter grew more abundantly with PR digests. All the strains of *B. breve*
290 and most of *B. longum* grew more abundantly in the media containing the PR digests than in the control. On
291 the contrary, most *B. bifidum* and *B. animalis* grew more abundantly using the control peptone as nitrogen
292 source than PR digests. *L. casei* grew more abundantly on PR digests, while *L. paracasei* 2306 and the *L.*
293 *rhamnosus* strains 2323, 2303, 2302, 2409 grew better on the control peptone. A trend common to all the
294 tested species and strains, with respect to the growth and the age of PR, could not be found. However, most
295 of the bifidobacteria that preferred PR digests over the control peptone grew more abundantly on PR36 or
296 PR24 than on PR16. On the other hand, most of the lactobacilli preferring PR digests over the peptone, grew
297 better on PR16 or PR24 than on PR36.
298
299 *3.3 Consumption of PR peptides by bifidobacteria and lactobacilli*
300 Peptide utilization was analyzed by UPLC/ESI-MS for 8 *Bifidobacterium* and 7 *Lactobacillus*
301 strains, representative of different species/subspecies and selected among those with best growth on PR

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 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). Moreover, it was the sole strain able to consume specific peptides, such as P14, P25, P29, P47, P48, P66, 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,

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* subsp. *infantis* WC 0438 (B7) was able to consume also P2, P7, P23, P52, P57, P59, P62, P63, P64, and P71. This explains the strains not grouping together, with a clear separation along PC1, PC2, (Fig. 5a) and PC4 (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 components and slightly differentiating only along PC4 (Supporting Information: Fig. S1).

Lactobacilli were less performing in PR peptide consumption compared to bifidobacteria (Fig. 3). They all consumed P1, P4, P9, P12, P18, and P20, but generally did not use, or used at lower extent, the vast majority of the other peptides. The first 2 PCs of the PCA model calculated on lactobacilli (accounting for 61% of total data variance) showed that the pattern of peptide consumption was homogenous for most of the 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 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 (L6) and *L. paracasei* 2306 (L7).

All the other lactobacilli clustered together at high values of PC1 and presented a similar pattern of peptide utilization. *L. rhamnosus* 2232 (L1), *L. rhamnosus* 2325 (L2), *L. rhamnosus* 2362 (L3), *L. casei* 2240 (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.

3.4 Microbiota cultures

Cultures of intestinal microbiota were performed in a medium containing 5 g/L of PR24 digest as only nitrogen source or peptone as control (Fig. 6). In both the cultures, total bacteria similarly increased by 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 Log_{10} cells/mL at the beginning of batch process, accounting for 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 control cultures, respectively. In control cultures bifidobacteria remained stable, representing approx. the 5% of eubacteria. In PR24 cultures, they increased up to 8% after 12 h and 14% after 24 h, even though in this latter time-point the higher abundance arose also from the slight decrease in total bacteria. Unlike bifidobacteria, lactobacilli never appeared above the limit of detection of 4 Log_{10} cells/mL. Most of the peptides of PR24 digest were consumed in the first 6 h of incubation and completely disappeared in the following incubation hours (Fig. 6B).

4 Discussion

The present study aimed to characterize the peptides that are expected to escape digestion of PR and 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 peptides of 2 to 103 residues originated during ripening through the proteolytic cleavage of cheese proteins (mainly α and β-casein) by rennet, SLAB, and NSLAB (McSweeney, 2004). The water extracts of PR16, PR24, and PR36 shared similar profile, consistently with previous evidence that the most relevant proteolytic transformations occur in the first 12 months, before the PR can be sold on the market (Sforza et al. 2008).

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 a thorough characterization of the whole profile. The main peptide composition of PR after simulated digestion is reported herein for the first time. The PR digests lacked serum proteins and any peptide longer 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 predictable and specific cleavage sites could be found in peptide sequences, consistently with the fact that the proteolytic activity of pancreatin is due to trypsin, chymotrypsin, but also elastase, carboxypeptidase and other minor proteases, and suggesting that many peptides are generated by the action of an exopeptidase or 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–

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 sustained abundant growth of pure cultures of bifidobacteria and lactobacilli, natural beneficial colonizers of the human colon and potential probiotics (Rossi and Amaretti, 2010; Walter, 2008). Bifidobacteria were more efficient in consuming PR peptides than lactobacilli, with a greater number of peptides consumed at a greater extent. Peptide preferences of bifidobacteria were specific for each strain, as evidenced by PCA, with a core of peptides with 4 or 5 residues consumed by all strains (P1, P27, P28, P30, and P45). On the other 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 to a limited number of peptides, such as P64, P68, and P72. The lower peptides consumption by lactobacilli is consistent with the ability of NSLAB to use also free amino acids and other nitrogen compounds derived 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 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 of sulfur-containing amino acids, or the abundance of residues with peculiar properties (hydrophobicity, polarity, charge) and likely depend on specific proteases and/or peptide transporters preferentially recognizing specific sequence motifs in the peptides.

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

in rapid and complete consumption of peptides in the first hours of incubation, confirming the intense breakdown and assimilation of peptides by the dense proteolytic population of the colon (Richardson et al., 2013). Unlike bifidobacteria, the population of lactobacilli never increased in microbiota cultures over the limit of detection of qPCR quantification (4 Log10 cells/mL), likely because resident lactobacilli represent a minority population within the human gut microbiota (Quartieri et al., 2016; Rossi et al., 2016).

5 Conclusions

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 of PR digests is attractive and deserves further studies. Despite the limitation of the experimental procedure, that did not take into account the absorption in the small intestine, it was demonstrated that PR digests 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 supplementation, even though the highly proteolytic environment and the strong and unpredictable competition among several other bacteria make impossible to predict what can happen *in vivo*. The 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 composition and deserves deeper investigation.

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578 **Figure legends**

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

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

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

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

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

596 **Figure 6.** Cultures of human intestinal microbiota with PR24 digests. **Panel A:** time-course of total
597 eubacteria and bifidobacteria in FM medium supplemented with 5 g/L of PR24 digests (orange) or control
598 peptone (grey). Bacteria were quantified by qPCR. Symbols: total eubacteria, dotted line; bifidobacteria,
599 dashed line; relative amount of bifidobacteria in the microbiota, solid line. Values are means ± SD, n = 3.
600 **Panel B:** Overlaid chromatograms of PR24 cultures of intestinal microbiota at 0 (black), 6 (green), and 12 h
601 (yellow) of incubation. Chromatogram was obtained by UPLC/ESI-MS and they are showed as Total Ion
602 Current (TIC); run time: 72 min.

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

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Abstract

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, some of which have potential healthy bioactive properties. This study aimed to: i) determine the peptide profile of PR cheese subjected to simulated gastrointestinal transit; ii) evaluate *in vitro* whether the peptides could support growth of beneficial microbial groups of the gut microbiota. PR samples were subjected to *in vitro* digestion, simulating oral, gastric, and duodenal transit. Liquid chromatography coupled with tandem mass spectrometry revealed that digestion caused the disappearance of the serum proteins and most of the 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), and to bioreactor batch cultures of human gut microbiota. Most of bifidobacteria and lactobacilli grew more abundantly on PR digests than on the control peptone, and exhibited strain- or species- specific peptide preferences, as evidenced by principal component analysis. Bifidobacteria generally consumed a greater amount of peptides than lactobacilli, in terms of both the mean peptide consumption and the number of peptides consumed. For bifidobacteria, peptide preferences were very diverse, but a core of 10 peptides with 4 or 5 residues were consumed by all the strains. Lactobacilli behaved more homogenously and consumed nearly only the same 6 peptides, mostly dipeptides. The peptide preferences of the different groups of 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 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 bifidobacteria. This study demonstrated that peptides derived from simulated gastrointestinal digestion of PR supported the growth of most lactobacilli and bifidobacteria.

Keywords

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

Abbreviations

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

1 Introduction

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 and natural whey starter and a Protected Designation of Origin, in compliance with the European norm currently in force (European Commission, 2009, 2011). The curd is heated at 55°C to select thermophilic 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 fractions, the ratio of fat to protein being around 0.94, depending on milk characteristics. Ripened PR is 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 mg) acids (Gatti et al., 2014). The overall mineral content of PR is 4.0 to 4.5%, with NaCl at approximately 1.5% (Gatti et al., 2014).

Long ripening times allow for cheese extensive proteolysis (Fox and McSweeney, 1998; McSweeney, 2004; Visser, 1993) due to the residual rennet activity and the enzymes of starter (SLAB) and non-starter (NSLAB) lactic acid bacteria. During ripening, proteolysis gives rise to the continuous evolution 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 improved digestibility, reduced allergenicity, and flavor development (Alessandri et al., 2012; Sforza et al., 2012). The potential biological activity of PR peptides has recently attracted particular interest (Korhonen et al., 2009; Tidona et al., 2009). *In vitro* studies revealed calcium binding properties (Kim et al., 2004; Pinto et al., 2012) and antioxidant (Bottesini et al. 2013; Gupta et al., 2009), antihypertensive (Bernabucci et al., 2014), antimicrobial activities (Benkerroum, 2010; Rizzello et al., 2005)..

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

77 dismicrobism, or post-infective conditions (Olivi et al., 1979; Pancaldi et al., 2008). In particular, a
78 homemade food based on PR cheese was developed and successfully utilized to feed infants suffering from
79 different forms of intestinal problems, with a rapid clinical improvement and normalization of the gut. The
80 high digestibility and the high amounts of short chain fatty acids, amino acids, and oligopeptides easily
81 absorbed in the bowel likely support this beneficial effect. Furthermore, the hypoallergenicity, the absence of
82 lactose, and the high sodium content that restores losses of salts and reduces the secretions of intestinal cells
83 have been claimed as responsible of the positive outcome of this dietary therapy (Olivi et al., 1979; Pancaldi
84 et al., 2008).
85 The human colon is colonized by a dense and complex bacterial community exerting important
86 effects on the health status (Sekirov et al., 2010), wherein the commensal bifidobacteria and lactobacilli are
87 recognized as beneficial and are commonly utilized as probiotics (Rossi and Amaretti, 2010; Walter, 2008).
88 Previous studies demonstrated that the hydrolysates of proteins from different origins, and particularly from
89 milk, can act as growth factors for lactobacilli or bifidobacteria (Poch and Bezkorovainy, 1991, Ibrahim and
90 Bezkorovainy, 1994; Liepke et al., 2002; Oda et al., 2013; Meli et al., 2013, 2014). Moreover, the peptides
91 released with the gastric digestion of an aged cheese were demonstrated to modulate the composition of mice
92 microbiota *in vitro*, improving the abundance of bifidobacteria (Condezo-Hoyos et al., 2016). On this basis it
93 has been hypothesized that PR peptides could promote growth and/or activity of these beneficial microbes,
94 thus modulating the resident microbiota towards a balanced and healthy composition (Sekirov et al., 2010).
95 The health effects of PR bioactive peptides on intestinal bacteria require that they resist
96 gastrointestinal digestion and, at least partially, reach the colon. The present study aimed to characterize the
97 peptides derived from simulated gastrointestinal digestion of PR cheese, utilizing an *in vitro* digestion model
98 that recently achieved an international consensus (Minekus et al., 2014). The resulting peptide mixtures were
99 characterized by liquid chromatography coupled with mass spectrometry. In order to verify whether peptides
100 specifically promoting the growth of beneficial bacteria and potential probiotics could be identified, the
101 misxtures were tested as nitrogen source for bifidobacteria and lactobacilli in pure and fecal cultures.

2 Materials and Methods

2.1 Cheese water soluble extract

Samples of PR aged 16, 24, and 36 months (hereinafter referred to as PR16, PR24, and PR36, respectively) were provided by Consorzio del Formaggio Parmigiano Reggiano (Modena, Italy). Five grams of finely ground cheese were added with 45 mL of HCl 0.1 M and 1.25 mL of an aqueous solution of 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 proteins were removed by acid precipitation followed by centrifugation (45 min at 4°C at 3220 × g), according to Hernàndez-Ledesma et al. (2012). The supernatant was filtered through paper filter and extracted three times with diethyl ether to remove fats. Ether residues were removed with a rotavapor, and the residual solution was filtered subsequently through 5 µm and 0.45 µm filters.

2.1 Simulated gastrointestinal digestion of PR samples

All the chemicals were purchased from Sigma-Aldrich (Steinheim, Germany) unless otherwise stated. The cheese was finely grounded and digested following the procedure, consisting in three main steps: 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 KH_2PO_4, 13.6 mM $NaHCO_3$, 0.15 mM $MgCl_2$, and 0.06 mM $(NH_4)_2CO_3$), 2.5 mL of 1500 U/mL amylase, 125 µL of 300 mM $CaCl_2$, and 4.875 mL of distilled water (ratio cheese:digestive fluid of 1:1, w:v). The sample was vortexed and incubated for 2 min at 37°C on a reciprocating shaker. Then, 37.5 mL of gastric buffer (6.9 mM KCl, 0.9 mM KH_2PO_4, 25 mM $NaHCO_3$, 47.2 mM NaCl, 0.1 mM $MgCl_2$, and 0.5 mM $(NH_4)_2CO_3$), 8 mL of 25000 U/mL pepsin, 25 µL of 300 mM $CaCl_2$, 1 mL of 1 M HCl and 3.475 mL of water were added (final ratio cheese:digestive fluids 1:3, w:v). 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 KH_2PO_4, 85 mM $NaHCO_3$,

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 µL of 300 mM $CaCl_2$, 750 µL of 1 M NaOH and 6.55 mL of distilled water were added (final ratio cheese:digestive fluids 1:7, w:v). The pH was adjusted to 7 using 1 M NaOH. The sample was vortexed and incubated for 2 hours at 37°C on a reciprocating shaker. To stop the digestion the sample was heated at 95°C for 15 min, then cooled. Since the assessment of digestion is strongly affected by the analysis of the digested fraction, the samples were centrifuged for 45 min at 4°C at 3220 × *g* to remove insoluble proteins and undigested components (Minekus et al., 2014). The supernatant was filtered through 0.45 µm membranes. For chromatographic analysis, 196 µL of sample was supplemented with 4 µL of 1 mM Phe-Phe.

2.2 HPLC-ESI-MS/MS analysis.

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

Peptides were manually identified as follows. Molecular ions were recorded from the Full Scan 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 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 pattern of these sequences was determined (http://db.systemsbiology.net/proteomicsToolkit/FragIonServlet.html last accessed on September 2016) and compared with the MS/MS spectra. The peptide sequences were assigned on the basis of the matching fragments.

2.3 UPLC/ESI-MS analysis.

Absolute quantification of all the peptides identified was not possible due to the unavailability of all 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 performed using Phe-Phe as internal standard. The ratio between the chromatographic peak of the peptide and that of Phe-Phe did not yield absolute peptide concentration but allowed the comparison of the same 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 separated by a reverse phase column (Acquity UPLC BEH 300 C18, 1.7 μm, 2.1 × 150 mm equipped with a Acquity UPLC BEH C18 VanGuard Pre-column, 300Å, 1.7 μm, 2.1 × 5 mm, Waters) in an UPLC system coupled with ESI and MS (UPLC Acquity with a single quadrupole detector SQD, Waters). In order to replicate a chromatographic trace similar to HPLC, but with shorter run time, gradient elution with the same eluents was programmed as follows: 0-7 min, 100% A; 7-50 min, from 100% A to 50% A; 50-52.6 min, 50% 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, 100% A. The analysis parameters were: flow 0.2 mL/min; analysis time 72 min; column temperature 35°C;

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

2.4 Strains and culture conditions

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

2.5 Pure culture conditions

Utilization of PR digests was investigated in a basal medium (hereinafter referred to as BM) where PR16, PR24, PR36 digests or Bacto peptone (BD Difco) were utilized as nitrogen source, the latter as positive control. The medium contained lactose, 10 g/L; PR hydrolysates or Bacto peptone (BD Difco), 5 g/L; ammonium citrate, 2 g/L; yeast nitrogen base w/o amino acids and ammonium sulfate (BD Difco), 1.7 g/L; polysorbate 80, 1 g/L; sodium acetate trihydrate, 5 g/L; K_2HPO_4, 2 g/l; $MgSO_4 \cdot 7H_2O$, 0.1 g/l; $MnSO_4 \cdot H_2O$, 0.05 g/L; cysteine hydrochloride, 0.5 g/l; pH was corrected to 6.5. Bacteria inoculated (5% v/v) in BM based media were incubated 24 h at 37°C, and passaged at least three times before evaluation of growth and peptide consumption.

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
204 diluted 1:100 in Ringer's solution (Oxoid, Basingstoke, UK), then 0.1 ml were used to inoculate tubes
205 containing 6 mL of BM media. The impedance measurement was performed at 37°C. Bacteria growth was
206 measured as E%, where E-value is the impedance change revealed by the system at the electrode surface.
207 Variation in electrical conductivity, monitored during time, is proportional to the change in the number of
208 microorganisms (Bancalari et al., 2016). Maximum value of E% was recorded as index of maximum
209 microbial growth.
210
211 *2.6 Cultures of intestinal microbiota*
212 Batch cultures of fecal microbiota were performed in bioreactors containing 200 mL of FM medium,
213 developed from the media described by Walker et al. (2005) and Duncan et al. (2002), with some
214 modifications: beech wood xylan, 0.6 g/L; citrus pectin, 0.6 g/L; maize amylopectin, 0.6 g/L; larch wood
215 arabinogalactan, 0.6 g/L; potato starch, 3 g/L; fructans (Synergy1, Beneo, Mannheim, Germany), 3 g/L;
216 peptone, 5 g/L; ammonium citrate, 2 g/L; KH_2PO_4, 2 g/L; NaCl, 4.5 g/L; $MgSO_4 \cdot 7H_2O$ 0.5 g/L; $CaCl_2 \cdot$
217 $2H_2O$ 0.045 g/L; $FeSO_4 \cdot 7H_2O$, 0.005 g/L; hemin, 0.01 g/L; bile salts (Oxgall, BD Difco) 0.05 g/L,
218 resazurin, 0.6 mg/L; reducing solution, 40 ml/L; mineral solution, 2 ml/L; vitamin solution, 1.4 ml/L.
219 Reducing solution contained: cysteine hydrochloride, 12.5 g/L; $NaHCO_3$, 80 g/L. Mineral solution
220 contained: EDTA, 500 mg/L; $FeSO_4 \cdot 7H_2O$, 200 mg/L; $ZnSO_4 \cdot 7H_2O$, 10 mg/L; $MnCl_2 \cdot 7H_2O$, 3 mg/L;
221 H_3BO_3, 30 mg/L; $CoCl_2 \cdot 6H_2O$, 20 mg/L; $CuCl_2 \cdot 2H_2O$, 1 mg/L; $NiCl_2 \cdot 6H_2O$, 2 mg/L; $NaMoO_4 \cdot 2H_2O$, 3
222 mg/L. Vitamin solution contained: menadione, 1 g/L; biotin 2 g/L; calcium pantothenate 2 g/L; nicotinamide,
223 10 g/L; vitamin B12, 0.5 g/L; folic acid 0.5 g/L; thiamine, 4 g/L; PABA, 5 g/L. Reducing, mineral and
224 vitamin solutions were filter sterilized and added to the autoclaved medium. PR24 digest or Bacto peptone
225 were utilized as nitrogen source at the concentration of 5 g/L in parallel batches, carried out in duplicate. The
226 inoculum was prepared pooling equal amounts of fecal microbiota from three healthy adult subjects. The

feces were homogenized (5% w/v) with the medium in the anaerobic cabinet (Anaerobic System, Forma Scientific, Marietta, OH) under a 85% N_2, 10% CO_2, 5% H_2 atmosphere. 10 mL of the suspension were inoculated into bioreactors (Sixfors V3.01, Infors, Bottmingen, Switzerland) each containing 190 mL of fresh sterile medium, and the processes proceeded for 48 h (37°C, CO_2 atmosphere, pH 6.2, stirring 60 rpm). 5 mL samples were collected at 0, 6, 12, 24 h and stored at -20°C until analyzed.

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

2.7 Principal component analysis of peptide consumption

Principal component analysis (PCA) (Massard et al., 1997; Wold et al., 1987) was carried out to 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 the extracts. The experimental data were analyzed using PLS Toolbox software ver. 8.1.1 (Eigenvector Research Inc., Wenatchee, WA, USA) for the calculation of PCA models. A PCA model was calculated on a dataset with size {45, 63} including all the 45 samples (i.e. 15 strains × 3 ripening ages of PR) obtained by digestions carried out with bifidobacteria and lactobacilli, each one characterized by the consumption values of 63 peptides. Two further PCA models were calculated considering bifidobacteria and lactobacilli separately, i.e. on two separate datasets with size equal to {24, 63} and to {21, 63}, respectively. Before

252 calculating the PCA models, the variables were pre-processed using mean centering. The number of
253 significant PCs, i.e., the number of PCs bearing useful information, was selected based on the scree plot
254 reporting the percentage of variance explained by each PC vs. the PC number.
255
256 **3 Results**
257 *3.1 Effects of simulated digestion on the peptide profile of PR*
258 The peptide profile of PR16, PR24, and PR36 was characterized by HPLC/ESI-MS/MS before and
259 after the *in vitro* simulated gastrointestinal digestion (Figure 1A, 1B). Intact caseins are not taken into
260 account, since they were removed from both the water soluble extracts and from the digested samples.
261 Before the simulated digestion, a common peptide profile was detected in the water extracts of PR16, PR24,
262 and PR36 samples (Fig. 1A). 63 main peptides were identified, including some N-lactosyl amino acids, with
263 a length up to 103 residues and a molecular weight spread over a wide range (Supporting Information: Table
264 S1). 31 peptides had a MW < 500 Da, 2 had a MW of 500-1000 Da, 12 fell in the range 1000-2000 Da.
265 Longer peptides were also identified, 12 with MW of 2-5 kDa and at 6 with MW of 5-20 kDa. Moreover, the
266 two isoforms of β-lactoglobulin (A and B) were clearly detectable at 40.14 min.
267 After *in vitro* digestion, the mixtures of PR16, PR24, and PR36 still shared similar profile. The
268 digests lacked intact proteins, including β-lactoglobulins, and 61 out of the 63 peptides identified in water
269 soluble extracts were no longer detectable, being degraded into shorter ones (Fig. 1B). The sole exception
270 were FVAPFPEVFGK and the phosphopeptide LEELNVPGEIVESLSSS. The former was present in the
271 water soluble extract, especially of the PR36, and remained in similar amounts after digestion, whereas the
272 latter occurred in the digests in minor amount. N-lactosyl amino acids also resisted digestion, but their
273 chromatographic signal was negligible, compared with those of peptides.
274 As a whole, 72 main peptides were identified in the PR digests (Supporting Information: Table S2)
275 and, with the exception of P3, P13, P71, and P72, were assigned to specific sequences. Compared with the
276 undigested water extracts, molecular weight distribution of the peptides was shifted toward lower MW: 12

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).

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. *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 *L. rhamnosus* were investigated for the ability to grow utilizing PR hydrolysates as nitrogen source. All the strains grew both in the control medium and the PR-based media (Fig. 2), exhibiting a species specific 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, while the strains belonging to the latter grew more abundantly with PR digests. All the strains of *B. breve* and most of *B. longum* grew more abundantly in the media containing the PR digests than in the control. On the contrary, most *B. bifidum* and *B. animalis* grew more abundantly using the control peptone as nitrogen 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 tested species and strains, with respect to the growth and the age of PR, could not be found. However, most of the bifidobacteria that preferred PR digests over the control peptone grew more abundantly on PR36 or PR24 than on PR16. On the other hand, most of the lactobacilli preferring PR digests over the peptone, grew better on PR16 or PR24 than on PR36.

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 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). Moreover, it was the sole strain able to consume specific peptides, such as P14, P25, P29, P47, P48, P66, 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,

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* subsp. *infantis* WC 0438 (B7) was able to consume also P2, P7, P23, P52, P57, P59, P62, P63, P64, and P71. This explains the strains not grouping together, with a clear separation along PC1, PC2, (Fig. 5a) and PC4 (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 components and slightly differentiating only along PC4 (Supporting Information: Fig. S1).

Lactobacilli were less performing in PR peptide consumption compared to bifidobacteria (Fig. 3). They all consumed P1, P4, P9, P12, P18, and P20, but generally did not use, or used at lower extent, the vast majority of the other peptides. The first 2 PCs of the PCA model calculated on lactobacilli (accounting for 61% of total data variance) showed that the pattern of peptide consumption was homogenous for most of the 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 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 (L6) and *L. paracasei* 2306 (L7).

All the other lactobacilli clustered together at high values of PC1 and presented a similar pattern of peptide utilization. *L. rhamnosus* 2232 (L1), *L. rhamnosus* 2325 (L2), *L. rhamnosus* 2362 (L3), *L. casei* 2240 (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.

3.4 Microbiota cultures

Cultures of intestinal microbiota were performed in a medium containing 5 g/L of PR24 digest as only nitrogen source or peptone as control (Fig. 6). In both the cultures, total bacteria similarly increased by 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 Log_{10} cells/mL at the beginning of batch process, accounting for 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 control cultures, respectively. In control cultures bifidobacteria remained stable, representing approx. the 5% of eubacteria. In PR24 cultures, they increased up to 8% after 12 h and 14% after 24 h, even though in this latter time-point the higher abundance arose also from the slight decrease in total bacteria. Unlike bifidobacteria, lactobacilli never appeared above the limit of detection of 4 Log_{10} cells/mL. Most of the peptides of PR24 digest were consumed in the first 6 h of incubation and completely disappeared in the following incubation hours (Fig. 6B).

4 Discussion

377 The present study aimed to characterize the peptides that are expected to escape digestion of PR and
378 to investigate whether they could support the growth of beneficial bacteria in the colon. The water soluble
379 extracts of PR contained a few intact proteins (e.g. the two isoforms of β-lactoglobulin) and a variety of
380 peptides of 2 to 103 residues originated during ripening through the proteolytic cleavage of cheese proteins
381 (mainly α and β-casein) by rennet, SLAB, and NSLAB (McSweeney, 2004). The water extracts of PR16,
382 PR24, and PR36 shared similar profile, consistently with previous evidence that the most relevant
383 proteolytic transformations occur in the first 12 months, before the PR can be sold on the market (Sforza et
384 al. 2008).
385 Few previous studies performed *in vitro* digestion of PR, but mainly focused on quantification of
386 specific peptides (Bordoni et al., 2011; De Noni and Cattaneo, 2010; Basiricò et al., 2015), without providing
387 a thorough characterization of the whole profile. The main peptide composition of PR after simulated
388 digestion is reported herein for the first time. The PR digests lacked serum proteins and any peptide longer
389 than 24 residues. The new peptides derived from the hydrolysis of the high molecular weight peptides
390 already present in cheese and from the breakdown of intact α- and β-casein and whey proteins. Any
391 predictable and specific cleavage sites could be found in peptide sequences, consistently with the fact that the
392 proteolytic activity of pancreatin is due to trypsin, chymotrypsin, but also elastase, carboxypeptidase and
393 other minor proteases, and suggesting that many peptides are generated by the action of an exopeptidase or
394 from aspecific cleavages.
395 Some peptides identified in the digests bear sequences of biological interest. Nonetheless, it is not
396 clear whether they could be effectively active, since the sequences were found within longer peptides for
397 which the biological activity has not been investigated so far. In particular, the sequences of the opioids β-
398 casomorphin 5 (YPFPG) and β-casomorphin 7 (YPFPGPI) and the ACE inhibitor peptide β-casomorphin 9
399 (YPFPGPIPN) were found within longer peptides originating from β-casein, such as P50, P56, and P58
400 (Nguyen et al, 2015). The sequences of the ACE inhibitor tripeptides VPP and IPP were similarly found
401 within longer peptides of the digests, such as P63, P69, and P70. The ACE inhibitor sequence α_{S1}-cn f(24–

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 sustained abundant growth of pure cultures of bifidobacteria and lactobacilli, natural beneficial colonizers of the human colon and potential probiotics (Rossi and Amaretti, 2010; Walter, 2008). Bifidobacteria were more efficient in consuming PR peptides than lactobacilli, with a greater number of peptides consumed at a greater extent. Peptide preferences of bifidobacteria were specific for each strain, as evidenced by PCA, with a core of peptides with 4 or 5 residues consumed by all strains (P1, P27, P28, P30, and P45). On the other 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 to a limited number of peptides, such as P64, P68, and P72. The lower peptides consumption by lactobacilli is consistent with the ability of NSLAB to use also free amino acids and other nitrogen compounds derived 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 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 of sulfur-containing amino acids, or the abundance of residues with peculiar properties (hydrophobicity, polarity, charge) and likely depend on specific proteases and/or peptide transporters preferentially recognizing specific sequence motifs in the peptides.

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

in rapid and complete consumption of peptides in the first hours of incubation, confirming the intense breakdown and assimilation of peptides by the dense proteolytic population of the colon (Richardson et al., 2013). Unlike bifidobacteria, the population of lactobacilli never increased in microbiota cultures over the limit of detection of qPCR quantification (4 Log10 cells/mL), likely because resident lactobacilli represent a minority population within the human gut microbiota (Quartieri et al., 2016; Rossi et al., 2016).

5 Conclusions

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 of PR digests is attractive and deserves further studies. Despite the limitation of the experimental procedure, that did not take into account the absorption in the small intestine, it was demonstrated that PR digests 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 supplementation, even though the highly proteolytic environment and the strong and unpredictable competition among several other bacteria make impossible to predict what can happen *in vivo*. The 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 composition and deserves deeper investigation.

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578 **Figure legends**

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

582 **Figure 2.** Growth of 27 strains of *Bifidobacterium* (**Panel A**) and 30 of *Lactobacillus* (**Panel B**) in BM
583 medium containing 5 g/L control peptone (grey) or PR the digests from PR16 (yellow), PR24 (orange), and
584 PR36 (red). Growth of bifidobacteria and lactobacilli is reported as the increase of turbidity (OD_{600}) and
585 impedance (E%), respectively. Values are means, n = 3, SD < 0.1. The box and whiskers plots (**Panel C**)
586 report, for each species or group of species/subspecies, the increase in the different media. Boxes indicate the
587 median and 25^{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
589 medium containing 5 g/L of PR16, PR24, and PR36 digests. Percentage consumption, relative to non-
590 inoculated media similarly incubated for 24 h at 37°C, are reported as colors ranging from the lowest
591 (deepest green) to the highest (deepest red) consumption. Values are means, n = 3, SD always < 7.

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

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

596 **Figure 6.** Cultures of human intestinal microbiota with PR24 digests. **Panel A:** time-course of total
597 eubacteria and bifidobacteria in FM medium supplemented with 5 g/L of PR24 digests (orange) or control
598 peptone (grey). Bacteria were quantified by qPCR. Symbols: total eubacteria, dotted line; bifidobacteria,
599 dashed line; relative amount of bifidobacteria in the microbiota, solid line. Values are means ± SD, n = 3.
600 **Panel B:** Overlaid chromatograms of PR24 cultures of intestinal microbiota at 0 (black), 6 (green), and 12 h
601 (yellow) of incubation. Chromatogram was obtained by UPLC/ESI-MS and they are showed as Total Ion
602 Current (TIC); run time: 72 min.

Figure 1

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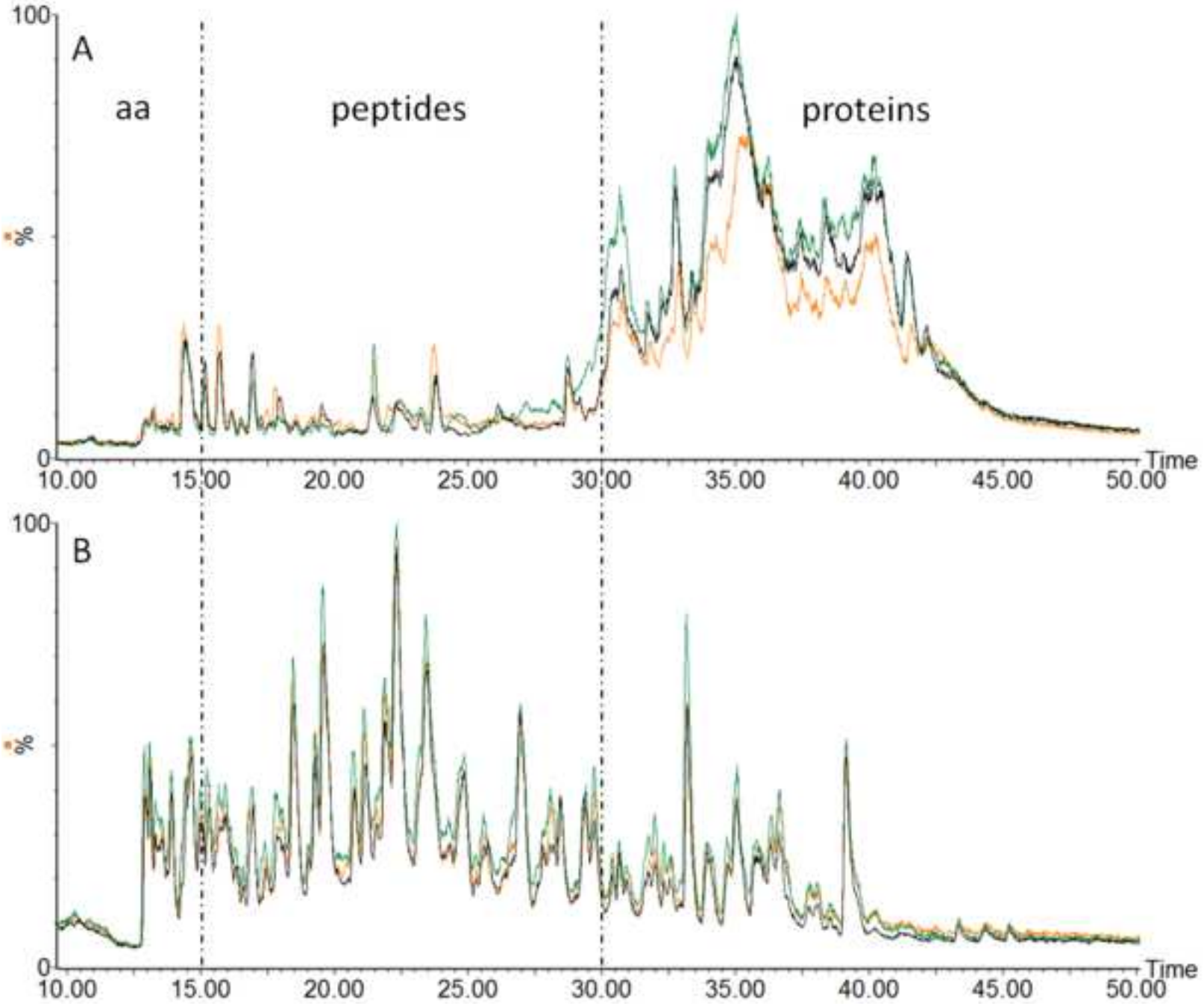

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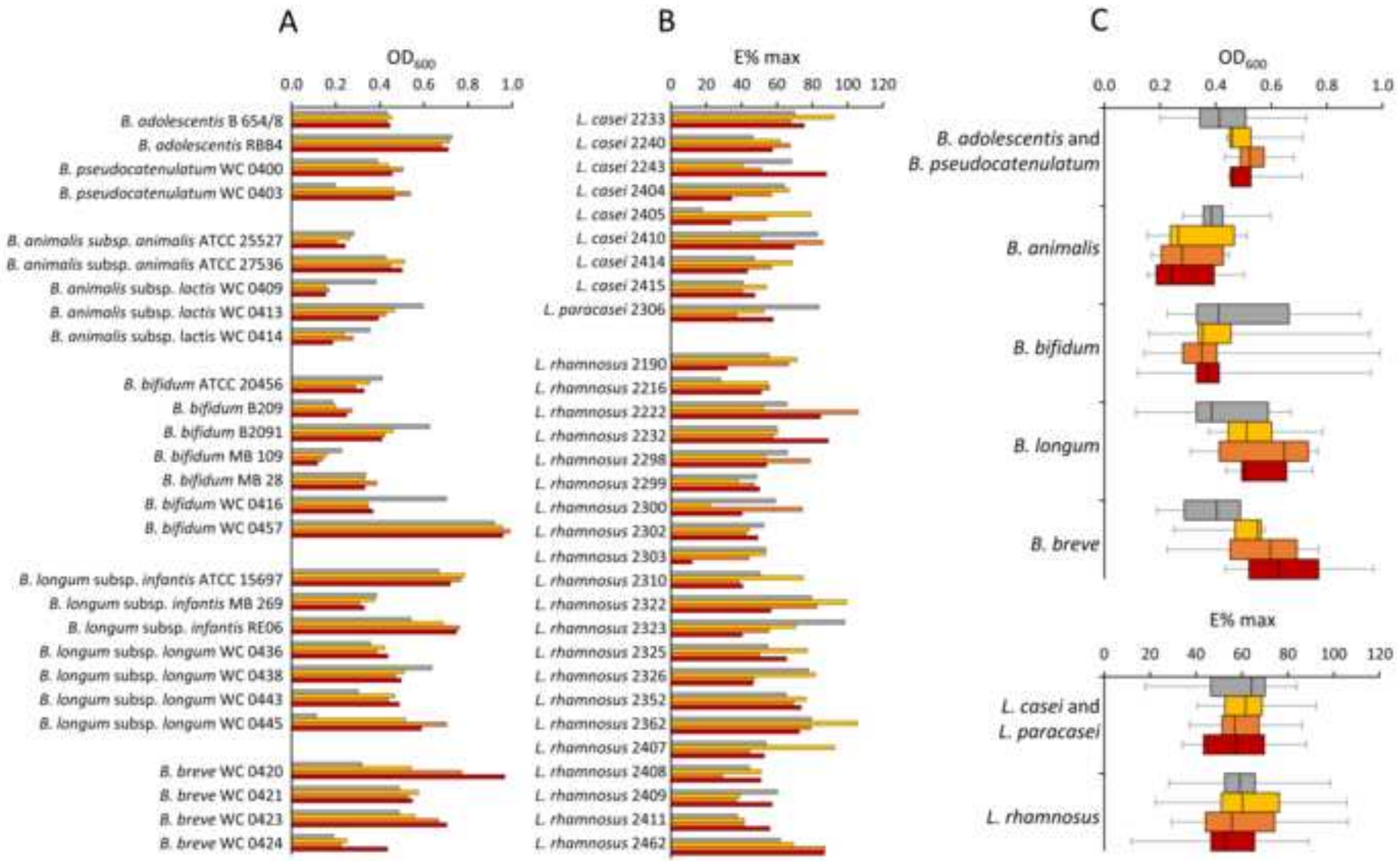

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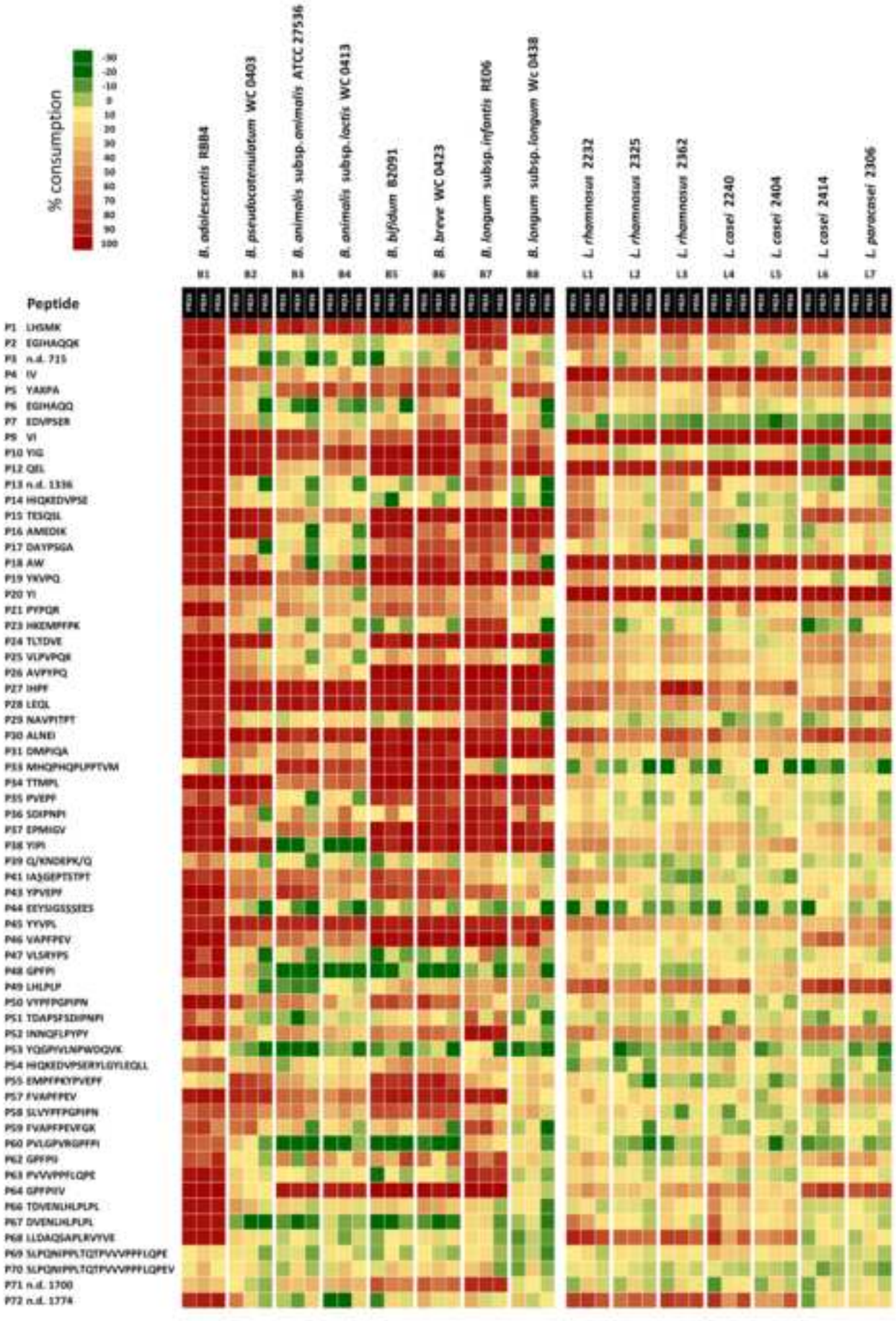

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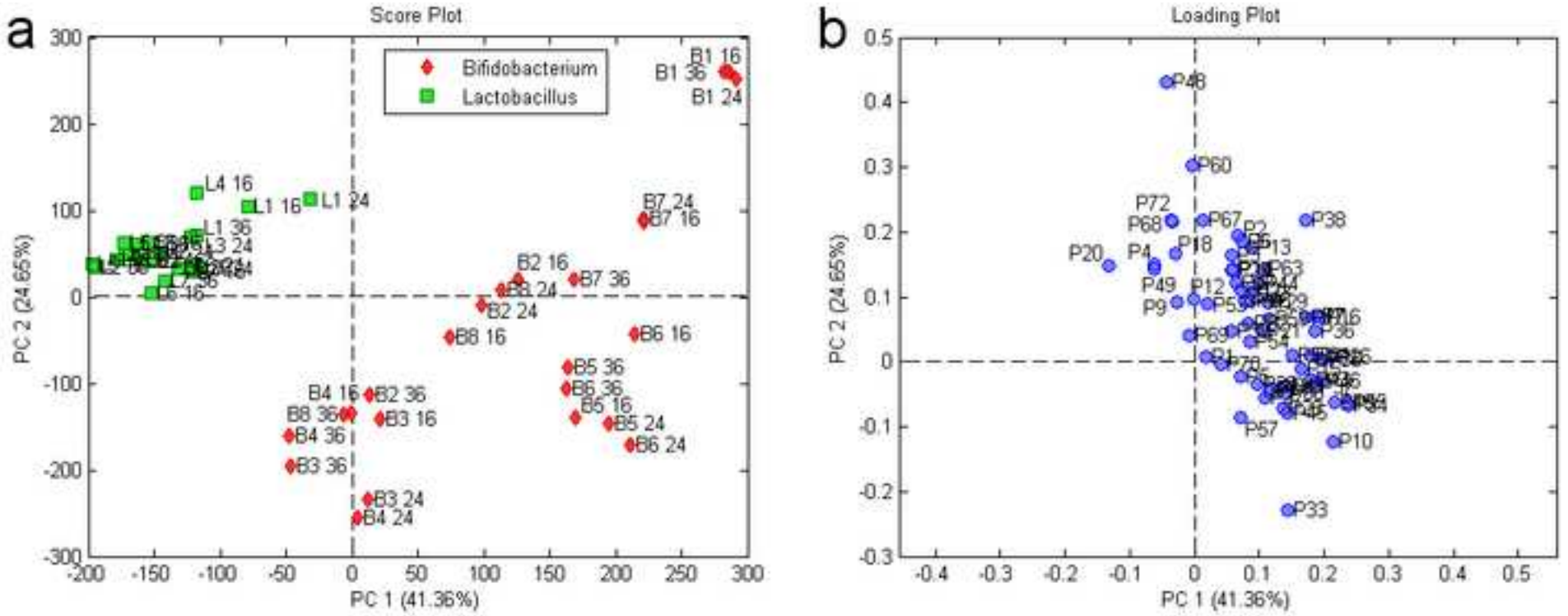

Figure 5

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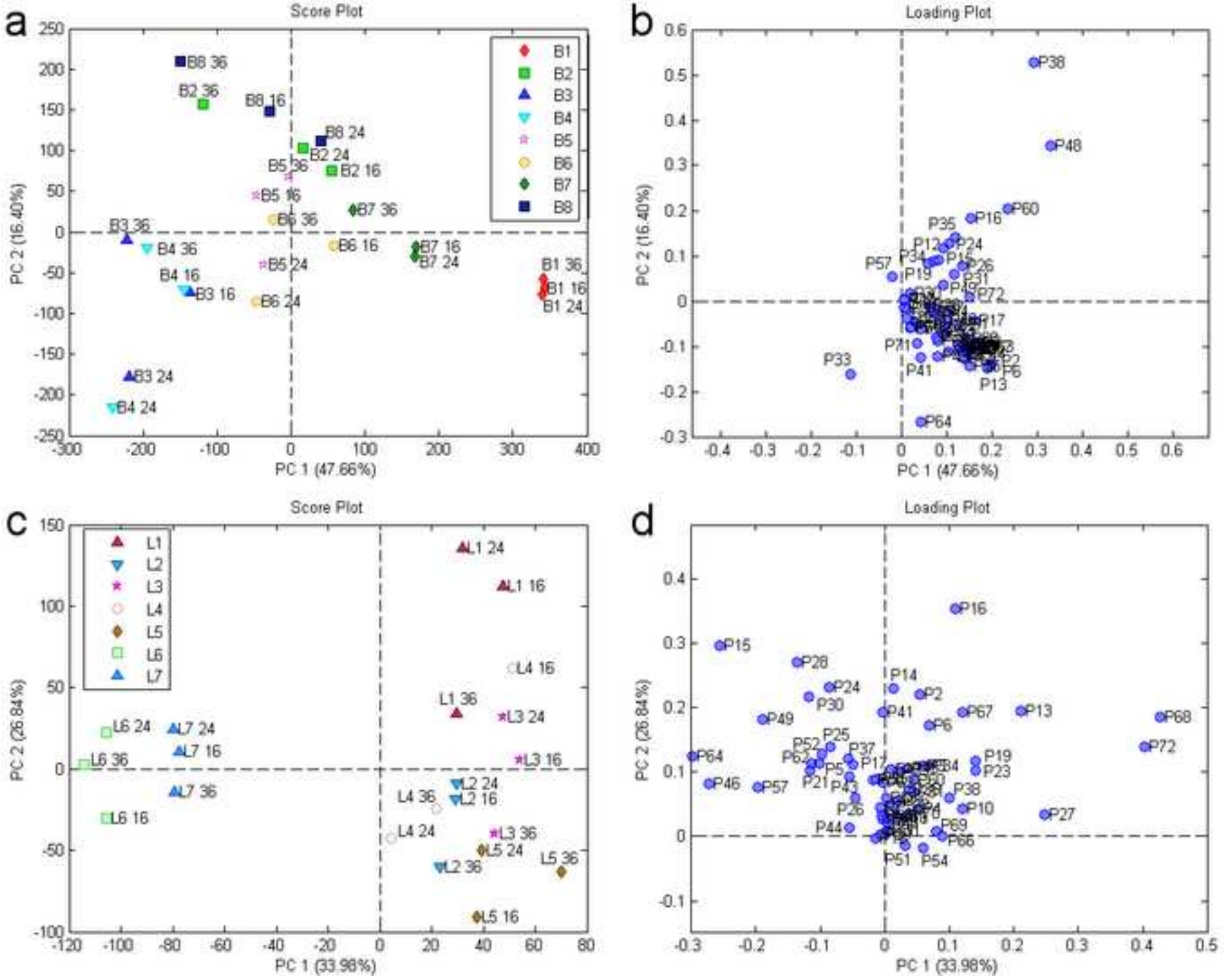

Figure 6

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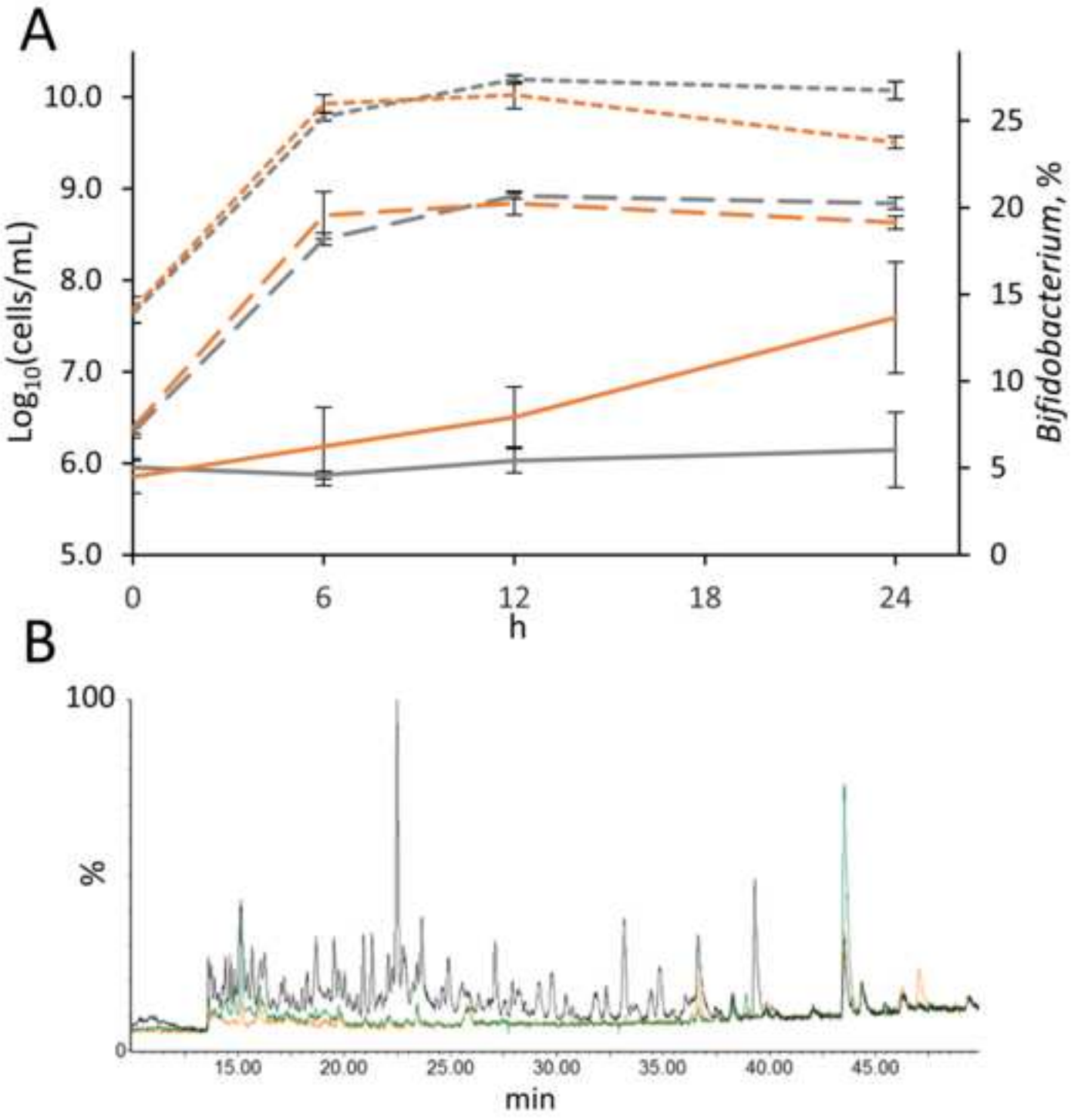

Supporting Information Table S1

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

$r_{T\ (min)}$	MW	SEQUENCE	a	b	c	x	y	z	PROTEIN
12.3	278	**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)	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	**SSSEESITR**							
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	**LSSSEESITR**	86 (1)				605.2 (5); 476.3 (4); 276.1 (2)		β-casein
34.2	571	**EIVPN**					230.1 (2); 329.1 (3)		α_{S1}-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	**SSEEIVPN**	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_{S1}-casein
36.8	244	**IL**	86.0 (1); 199.0 (2)	227.1 (2)	131.6 (1)		132 (1)		
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+); 536 (14, 3+)		α_{S2}-casein
40.1	203	**N-lactosyl-Ile/Leu**							
40.2	1999	**ESLSSSEESITRINK**	101.9 (1)	628.7 (9, 2+)			530.8 (4); 416.9 (7, 3+); 530.8 (12, 3+)	357 (3); 563.4 (13, 2+); 796.4 (12, 2+);	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	**KHYQKALNEINQFYQK**					585.4 (4)		α_{S2}-casein
41.0	278	**LF**	86 (1)				166 (1)		
42.6	237	**N-lactosyl-Phe**	192.1 (1)	220.1 (1)			238.1 (1)		
43.5	2264	**PQLEIVPNSAEERLHSMKEG**	912.9 (16, 2+)				723.3 (19, 3+)	524 (14, 3+)	α_{S1}-casein
45	1938	**TQTPVVVPPFLQPEVMGV**		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	**EELNVPGEIVESLSSSEESITRINKKIEK**	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+); 1336.6 (35)	128.9 (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	**KEKVNELSKDIGSESTEDQAMEDIKQ**		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	**STEDQAMEDIKQMEAESISS SEEIVPNSVEQKH 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);		

							909.5 (8); 1162.6 (29, 3+)		
57.4	12262	**KHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQMEAESISSSEEIVPNSVEQKHIQKEDVPSERYLGYLEQLLRLKKYK**		129.2 (1)			1343.9 (33, 3+)		α_{S1}-casein
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	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	**MGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQPHQPLPPTVMFPPQSVLSLSQSK**	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	**SLVYPFPGPIPNSLPQNIPPLTQTPVVVPPFLQPEVM**	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+)	

Supporting Information Table S2

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

#	$r_{T\ (min)}$	MW	SEQUENCE	Precursor Ion	a	b	c	P	y	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)		κ-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+); 583.1 (10, 2+)	κ-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	**IASGEPTSTPT**	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	**EEYSIGSSSEES**	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); 658.3 (6)	181.2 (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)	676.5 (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.**								

Supporting Information Figure S1

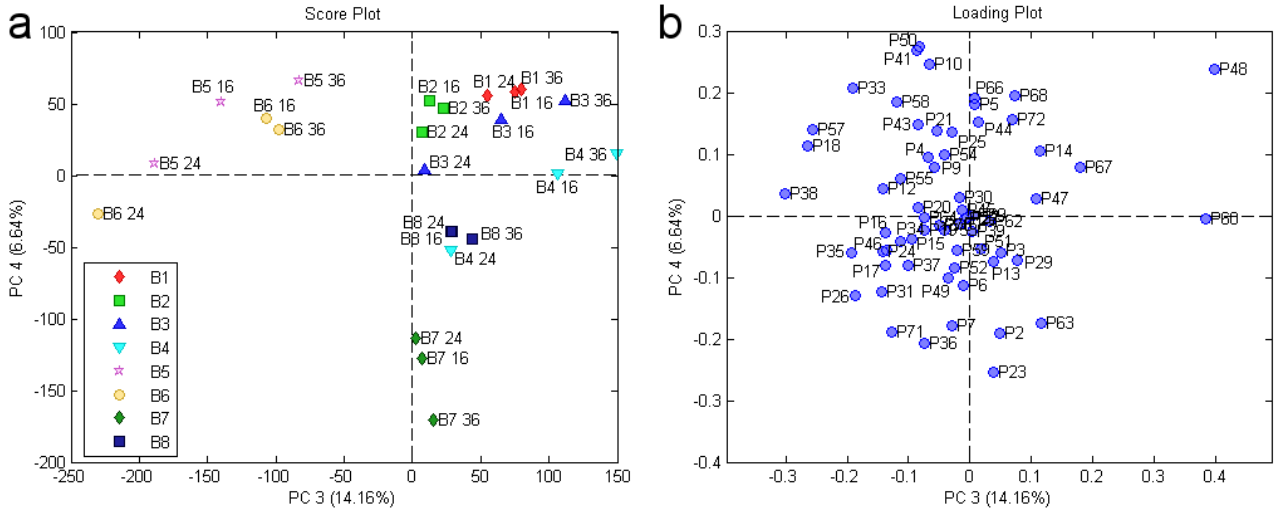

Figure S1. PCA model calculated on *Bifidobacterium* strains: PC3-PC4 score plot (**a**) and loading plot (**b**).