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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.

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

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

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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, 2009; Tidona et al., 2009). *In vitro* studies revealed calcium binding properties (Kim and Lim, 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 and Noratto, 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 mixtures 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.

2.2. 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 25,000 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 h 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.

2.3. 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