# Peptidomic study of casein proteolysis in bovine milk by *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331

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**Abstract**

Lactobacilli contain different cell envelope proteinases (CEPs) responsible for the hydrolysis of caseins and the release of various bioactive peptides. In this work, we explored the CEP activity of *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 whole cells towards β-, αS1-, κ- and αS2-caseins in bovine milk. Mass spectrometry analysis of fermented milk hydrolysates identified a total of 331 peptides, which were mainly derived from β-caseins (59.0 and 60.1% for PRA205 and PRA331, respectively). The analysis of αS1-casein (f1-23) cleavage site specificity congruently supports that *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 exhibited a mixed-type $CEP_{I/III}$ activity. PRA205 and PRA331 CEPs also showed cleavage site specificity toward β-casein, preferentially. These CEPs cleaved the peptide bond preferentially when hydrophobic or negatively charged amino acids were present. 13.5% and 13.7% of peptides released by *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 CEPs were found to have 100% homology with previously identified bioactive peptides.

## 1. Introduction

Food intake with the goal of improving human health is an ongoing focus for research. Recommendations for the consumption of certain nutritious fermented foods date back to the Hippocratic Corpus of Ancient Greece. The idea that lactic acid bacteria (LAB) fermenting milk are responsible for enhancing health and delaying the human aging was first proposed by the Russian scientist Elie Metchnikoff more than a century ago (Mackowiak, 2013). In the past years, research has documented a wide range of health benefits exerted by dairy LAB, especially immune and metabolic ones, and it is now focusing to decipher the microbial mechanisms underpinning these health-promoting effects (Reid, 2015).

Some beneficial effects exerted by LAB are due to the generation of secondary metabolites with health-promoting properties. The most important biogenic compounds in fermented milk are the bioactive peptides released from caseins via the LAB proteolytic system. Biological activities associated with such peptides include immunomodulatory, antibacterial, anti-hypertensive, antioxidant, mineral binding, and opioid-like properties (Brown et al., 2017). In addition, dairy LAB are auxotrophic for many amino acids and efficient casein breakdown is crucial to make LAB competitive as dairy starters (S-LAB), as well as suitable to survive in ripened cheeses as non-starter LAB (NS-LAB) (Kunji, Mierau, Hagting, Poolman, & Konings, 1996). The amino acid release also contributes to the aroma compound formation during cheese ripening and impacts sensorial properties and consumer's acceptance of dairy foods (McSweeney & Sousa, 2000).

Cell envelope proteinases (CEPs) are large multi-domain proteins anchored to the cell wall that catalyse the first step of hydrolysis of milk caseins into peptides. Different transport systems then internalize these peptides into the cell, where they are further hydrolysed by numerous intracellular peptidases (Savijoki, Ingmer, & Varmanen, 2006). Six different types of CEPs have been described in several LAB species: PrtB from *Lactobacillus delbrueckii* subsp. *bulgaricus* (Laloi, Atlan, Blanc, Gilbert, & Portalier, 1991); PrtH from *Lactobacillus helveticus* (Genay, Sadat, Gagnaire & Lortal, 2009); PrtL from *Lactobacillus delbrueckii* subsp. *lactis* (Villegas, Brown,

Savoy de Giori, & Hebert, 2015); PrtP from *Lactococcus lactis* (Kok, Leenhouts, Haandrikman, Ledeboer, & Venema, 1988), *Lactobacillus paracasei* (Holck & Naes, 1992), *Lactobacillus casei* (Fernández de Palencia, Peláez, Romero, & Martín-Hernández, 1997; Kojic, Fira, Banina, & Topisirovic, 1991), *Lactobacillus rhamnosus* (Guo et al., 2016) and *Lactobacillus plantarum* (Strahinic, Kojic, Tolinacki, Fira, & Topisirovic 2010); PrtR from *Lactobacillus rhamnosus* (Pastar et al., 2003); and PrtS from *Streptococcus thermophilus* (Siezen, 1999). These proteinases vary in substrate specificity, domain composition and cell wall anchoring, but all of them belong to the so-called subtilase family as they contain the catalytic serine protease domain showing sequence homology to the active site of subtilases (Savijoki et al., 2006; Sadat-Mekmene, Genay, Atlan, Lortal, & Gagnaire, 2011a). Most frequently, LAB possess only one CEP, but the presence of two CEPs has been described in lactobacilli (Sadat-Mekmene et al., 2011b).

Much of the current knowledge on LAB proteolytic system comes from studies on S-LAB species, such as *Lc. lactis*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus* and only few works has been done to elucidate the role of NS-LAB. Recently, the NS-LAB species *Lb. paracasei*, *Lb. casei* and *Lb. rhamnosus* were proven to generate bioactive casein-derived peptides during milk fermentation (Guo et al., 2016; Solieri, Rutella, & Tagliazucchi, 2015). *Lb. casei*/*Lb. paracasei* *PrtP*-encoded CEP was also demonstrated to degrade pro-inflammatory chemokines associated to inflammatory bowel diseases (Hormannsperger, von Schillde, & Haller, 2013). Consequently, there is an increasing interest to study NS-LAB proteases responsible for the release of bioactive peptides (Lozo et al., 2011).

In our previous work, we demonstrated that two mesophilic NS-LAB strains isolated from Parmigiano Reggiano ripened cheese, namely *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331, exhibit safety and technological performance compatible with probiotic properties (Solieri, Bianchi, Mottolese, Lemmetti, & Giudici, 2014). They also release the angiotensin-I-converting enzyme (ACE)-inhibiting peptides Valine-Proline-Proline (VPP) and Isoleucine-Proline-Proline (IPP) from caseins at doses that may exert antihypertensive effects *in vivo* (Solieri et al., 2015). Despite these

multiple interesting properties, the activity and specificity of CEPs from strains PRA205 and PRA331 remain unknown, as well as their potential to release additional milk-derived peptides other than VPP and IPP. The aim of this work was to fill this gap and to evaluate the pattern of casein breakdown by PRA205 and PRA331 whole cells CEP activities through a peptidomic approach.

## 2. Materials and Methods

### *2.1 Microorganisms, media and growth conditions*

*Lactobacillus casei* PRA205 and *Lb. rhamnosus* PRA331 were isolated from ripened Parmigiano Reggiano cheese (Solieri, Bianchi, & Giudici, 2012) and deposited in Unimore Microbial Culture Collection (www.umcc.unimore.it) for long-term preservation. The cultures were activated from their frozen forms (stored in MRS medium supplemented with 25% (v/v) glycerol at −80°C) by transferring them in MRS broth and incubating at 37°C for 24h under anaerobic conditions. After two rounds of growth on the same medium, strains were routinely maintained on MRS medium supplemented with 7% (w/v) agar at 4°C for the duration of the experiments.

### *2.2 Inoculum preparation and milk fermentation*

Milk fermentation trials were carried out in triplicate as follows. Single-colony cultures were inoculated in MRS broth for 24h at 37°C. Cells were washed twice with 50 mmol $L^{-1}$ Tris-HCl buffer (pH 6.5), re-suspended in 10% (w/w) skimmed milk and used as pre-cultures (2% v/v) to inoculate milk batches prepared with 50 mL of ultra-high temperature-treated (UHT) skimmed bovine milk. Fermentation was carried out for 72h at 37°C at 10 rpm. pH values were determined over time as previously reported (Solieri et al., 2015). At the end of the fermentation (pH values ≤ 4.0 for at least two consecutive measurements), samples were taken to estimate milk protein hydrolysis, ACE-inhibitory and radical scavenging activities as reported in section 2.4.

*2.3 Cell viability assay*

PRA205 and PRA331 cells were harvested by centrifugation after 24, 48 and 72h of milk fermentation, twice washed with physiological solution (9 g $L^{-1}$ NaCl) and re-suspended at the final concentration of $10^7$ CFU $mL^{-1}$, according to the correlation curves between $OD_{600nm}$ and CFU values previously established for every strain (Rutella, Tagliazucchi, & Solieri, 2016). Bacterial suspensions were stained with LIVE/DEAD BacLight Bacterial Viability Kit (Invitrogen) and live/dead cell ratio was measured according to manufacture instructions. Fluorescence intensity was measured with a Jasco FP-6200 spectrofluorometer (Jasco, Orlando FL, U.S.A.).

*2.4 Determination of milk protein hydrolysis, radical scavenging and angiotensin I-converting enzyme (ACE)-inhibitory activities*

Milk protein hydrolysis and radical scavenging activity were determined on the TCA-soluble supernatants (peptidic fractions) obtained by treating fermented milk with 1% (w/v) TCA followed by a centrifugation at 10,000*g* for 20 min (4°C). In particular, milk protein hydrolysis was determined by measuring the amounts of released amino groups using the 2,4,6-trinitrobenzenesulfonic acid (TNBS) assay (Adler-Nissen, 1979). Briefly, 50 µL of appropriately diluted peptidic fractions were mixed with 400 µL of sodium phosphate buffer (0.1 mmol $L^{-1}$; pH 8.2) and 400 µL of 0.1% TNBS solution (prepared in the same sodium phosphate buffer). After 60 min of incubation at 50°C, the reactions were stopped by adding 800 µL of HCl 0.1 mmol $L^{-1}$. The absorbance values at 340 nm were read using a Jasco V-550 UV/Vis spectrophotometer (Jasco, Orlando, FL, USA.). A calibration curve was prepared using leucine as standard (range 0.1-2.0 mmol $L^{-1}$) and the results were expressed as mmol L-1 of leucine equivalents.

The antioxidant activity of the peptidic fractions was measured as radical scavenging activity using the ABTS radical cation decolourization assay (Re et al., 1999) and expressed as mg $L^{-1}$ of Trolox.

ACE-inhibitory (ACEi) activity was determined according to Ronca-Testoni (1983) on the ultra-filtrated fraction obtained from fermented milk as previously reported (Solieri et al., 2015). The tripeptide 2-furanacryloyl–phenylalanylglycylglycine (FAPGG) was used as substrate assay and the ACEi activity was calculated as percent of inhibition (ACEi%).

Three analytical replicates were run for each sample collected at the end of each fermentation trial (carried out in triplicate) in all the assays.

*2.5 Determination of peptides with nanoflow LC-ESI-QTOF MS analysis*

Peptidomic analysis was performed by injecting the TCA-soluble supernatant of fermented milk on a 1200 Series Liquid Chromatographic two-dimensional system coupled to a 6520 Accurate-Mass QTOF LC/MS via a Chip Cube Interface (Agilent Technologies, Santa Clara, CA, USA) as described in Tagliazucchi, Helal, Verzelloni, Bellesia, & Conte (2016). Chromatographic separation was performed on a ProtID-Chip-43(II) including a 4 mm 40 nL enrichment column and a 43 mm × 75 μm analytical column, both packed with a Zorbax 300SB 5 μm C18 phase (Agilent Technologies). The mobile phase consisted of (A) $H_2O$/acetonitrile/formic acid (96.9:3:0.1, v/v/v) and (B) acetonitrile/$H_2O$/formic acid (94.9:5:0.1, v/v/v). The sample (2 μL) was loaded onto the Chip enrichment column at a flow rate of 4 μL $min^{-1}$ with a mobile phase consisting of 100% A using a G1376A capillary pump. A flush volume of 2 μL and a flush-out factor of 5 were used. After valve switching, a gradient elution was performed throughout the enrichment and analytical columns at 500 nL $min^{-1}$ using a G2226A nano pump. The gradient started at 0% B for 1 min, and then linearly ramped up to 90% B in 70 min. The mobile phase composition was maintained at 90% B for 15 min in order to wash both enrichment and analytical columns. The mass spectrometer was tuned and calibrated according to the manufacturer's instructions in extended dynamic range (2 GHz) mode as reported by Dei Più et al. (2014).

For peptide identification, MS/MS spectra were converted to .mgf files and were then searched against the Swiss-Prot database using Protein Prospector (http://prospector.ucsf.edu) and

MASCOT (Matrix Science, Boston, MA, USA) protein identification softwares. The following parameters were considered: enzyme, none; peptide mass tolerance, ± 40 ppm; fragment mass tolerance, ± 0.12 Da; variable modification, oxidation (M) and phosphorylation (ST); maximal number of post-translational modifications permitted in a single peptide, 4. We considered only peptides with a best expected value lower than 0.05 that corresponded to $P<0.01$. The assignment process was complemented and validated by the manual inspection of MS/MS spectra. Three replicates for each fermentation trial was injected in the mass spectrometer and only the peptides present in at least two replicates were considered significant and included in the analysis.

*2.6 Identification of bioactive peptides*

The identified peptides in milk samples were investigated for literature-identified bioactive peptides using the BIOPEP database and the Milk Bioactive Peptide Database (MBPDB) (Minkiewicz, Dziuba, Iwaniak, Dziuba, & Darewicz, 2008; Nielsen, Beverly, Qu, & Dallas, 2017). Only peptides with 100% homology to known functional peptides were considered as bioactive peptides.

The relative amount of the bioactive peptides was estimated by integrating the area under the peak (AUP). AUP was measured from the extracted ion chromatograms (EIC) obtained for each peptide and normalized to the peptide content of milk hydrolysates. The peptide content was determined at the end of the fermentation trials by using the TNBS method as described in section 2.4 and expressing the results as mg of leucine equivalent $mL^{-1}$

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*2.7 Calculation of the cleavage specificity*

The cleavage probability and positive or negative influence on the cleavage of an amino acid in the P1 and P1’ subsites were calculated according to Keyl (1992).

The subsite nomenclature was according to Schechter & Berger (1967) where the amino acid residues are designated as P1 in the N-terminal direction (on left of the sequence) and Pl’ in the C-terminal direction (on right of the sequence) from the cleaved bond. The subsite P1 interacts with

the subsite S1 in the enzyme active site, whereas the subsite P1' interact with the subsite S1' in the enzyme active site. Therefore, the peptidic bond cleaved by the protease was defined as the P1-P1' bond. We quantitatively analysed the influence of specific amino acid residues in position P1 or P1'on the CEP cleavage probability.

If the amino acid residue *A* is in the position *n* (P1 or P1' subsite), the cleavage probability of the P1–P1' bond will be:

$$\%Pn = \frac{total\ amino\ acid\ A\ cleaved\ in\ position\ n}{total\ amino\ acid\ A\ in\ proteins} \times 100$$

and in consequence the mean cleavage probability:

$$\%\overline{Pn} = \sum_{\#=1}^{20} \frac{\%Pn}{20}$$

The coefficient *Kn* was used to quantify the positive or negative influence of an amino acid residue *A* in the P1 and P1' subsites:

$$Kn = \frac{\%Pn}{\%\overline{Pn}} - 1$$

*Kn* values >0 indicated a positive influence of the amino acid *A* in the specific subsite on the cleavage of the P1-P1' bond, whereas *Kn* values <0 suggested a negative effect on the cleavage.

*2.8 Statistical analysis*

All data are presented as mean ± standard deviation (SD) for three replicates. The Student's t-test was performed using GraphPad Prism 6.0 (GraphPad Software, San Diego, CA, USA). The differences were considered significant with $P < 0.05$. Venn diagrams were drawn using the online tool VENNY 2.1.0 (Oliveros, 2015).

## 3. Results and Discussion

### *3.1 Characterization of fermented milk*

Analysis of CEP activities on purified caseins could tend to overestimate the true caseinolytic capability of whole cells towards casein micelles in milk (Sadat-Mekmen et al., 2011b). The use of purified CEPs instead whole-cell anchored CEPs may also modify the specificity of the proteinase towards caseins (Fernández de Palencia et al., 1997). Therefore, we decided to evaluate the CEP activities of whole cells of *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 towards the caseins in UHT milk.

Milk samples were inoculated with standardized amounts of PRA205 and PRA331 single cultures without any pre-adaptation step. After 72h of incubation, pH values were 4.00 in both sets of samples and remained stable over time. At the end of fermentation, strain PRA205 showed value of leucine equivalents of 10.93 ± 0.93 mmol $L^{-1}$, whereas PRA331 of 6.40 ± 0.92 mmol $L^{-1}$. These data agree with earlier results showing that PRA205 is more proteolytic than PRA331 towards milk caseins (Solieri et al., 2015). *Lb. casei* PRA205 produced milk hydrolysates with ACEi activity higher than that exhibited by milk hydrolysates with *Lb. rhamnosus* PRA331 (75.8 ± 3.2 *vs* 68.5 ± 2.6 ACEi%). Similarly, antioxidant activity was slightly higher in hydrolysates by strain PRA205 than the hydrolysates by strain PRA331 (249.12 ± 15.10 *vs* 202.57 ± 18.66 mg $L^{-1}$ of trolox, respectively).

The level of bacterial lysis during milk fermentation was monitored to exclude that the peptides could be generated by intracellular peptidases released into the hydrolysates. Cell viability was estimated app. 100% for both PRA205 and PRA331 after 24 and 48h of incubation (data not showed). Interestingly, at the end of milk fermentation the percentage of viable cells was 90.49 ± 0.74% and 94.59 ± 5.70% for PRA205 and PRA331, respectively. These data indicated that no significant lysis occurred during milk fermentation and supported that the observed casein

proteolysis was mainly due to the action of CEPs anchored on the whole cells rather than intracellular proteinases or peptidases.

*3.2. Peptidomic analysis of milk fermented with Lactobacillus casei PRA205 and Lactobacillus rhamnosus PRA331*

Mass spectrometry analysis was used to identify the full set of peptides present in milk hydrolysates by the selected strains. A total of 331 milk peptides were released by the CEPs activities of PRA205 and PRA331 whole cells. In particular, 178 peptides were identified in PRA205 samples (see supplementary online **Tables S1-S4** and **Figures S1-S4**) and 153 peptides in PRA331 samples (see supplementary online **Tables S5-S8** and **Figures S1-S4**).

The analysis of the identified sequences according to their protein of origin showed that the main identified peptides were derived from β-casein, which was the preferred substrate over αS1-, κ- and αS2-caseins. The β-casein-derived peptides were 59.0 and 60.1% of the total identified peptides in PRA205 and PRA331 samples, respectively, followed by αS1-casein-derived peptides (18.5 and 19.0% of the total identified peptides in PRA205 and PRA331 samples, respectively) and κ-casein-derived peptides (16.3 and 15.0% of the total identified peptides in PRA205 and PRA331 samples, respectively). PRA205 and PRA331 CEPs poorly hydrolysed αS2-casein, resulting in only 11 (corresponding to the 6.2% of total identified peptides) and 8 peptides (corresponding to the 5.9% of total identified peptides), respectively. As expected, no significant proteolysis of whey proteins was observed for both the strains. The Venn diagram (**Figure 1**) showed that 24.6 and 14.0% of peptides were specific for PRA205 and PRA331 milk hydrolysates, respectively. The majority of the identified peptides were found in both the milk hydrolysates, suggesting that PRA205 and PRA331 share a similar caseinolytic pattern.

CEPs are classified on the basis on their caseinolytic specificity (Kunji et al. 1996). Typically, two CEPs have been identified: a $P_I$-type, which preferentially hydrolyses β-casein, and a $P_{III}$-type, which acts on αS1-, β- and κ –caseins equally well (Pritchard & Coolbear, 1993; Visser,

Exterkate, Slangen & de Veer 1986). A third group, termed $P_I/P_{III}$-type has been described to classify intermediate proteases, capable to cleave β-casein like the $P_I$-type and, to a lesser extent, α- and κ-caseins (Exterkate, Alting, & Bruinenberg, 1993). Both *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 exhibited a predominant CEP activity towards β-casein, and a lower proteolytic activity towards α- and κ-caseins. Cell viability data allowed us to exclude that intracellular aminopeptidase released by lysed cells may significantly contribute to this pattern of casein breakdown. Furthermore, no extracellular aminopeptidases have been reported for *Lb. casei* and *Lb. rhamnosus* (Christensen, Dudley, Pederson, & Steele, 1999). Overall, these evidences support that the observed CEP activities could be due to the mixed $P_I/P_{III}$-type proteases. $P_I/P_{III}$-type proteases have been characterized in lactobacilli (Fernandez de Palencia et al., 1997; Sadat-Mekmene et al., 2011a Villegas et al., 2015) and lactococci (Nikolić, Tolinački, Fira, Golić, & Topisirović, 2009). In particular, like PRA331, *Lb. rhamnosus* BGT10 has PrtR protease suitable to cleave both β- and α-caseins.

*3.3. Analysis of the αS1-casein (f1-23) cleavage sites*

CEPs are commonly classified according to their specificities toward the αS1-casein fragment comprising residues from 1 to 23 (Exterkate, 1995). In strains PRA205 and PRA331, CEPs hydrolysed the αS1-casein (f1-23) fragment at the $H_8$-$Q_9$, $Q_9$-$G_{10}$, $Q_{13}$-$E_{14}$, $N_{17}$-$E_{18}$ and $L_{21}$-$R_{22}$ positions, respectively (**Figure 2**). In addition, PRA331 also cleaved at the $L_{16}$-$N_{17}$ position. The majority of these cleavage sites are typical of mixed $P_I/P_{III}$-type CEPs isolated from several lactococci, *S. thermophilus* CNRZ 385, *Lb. delbrueckii* subsp. *lactis* CRL 581 and *Lb. helveticus* L89 (Exterkate, 1995; Fernandez-Espla, Garault, Monnet, & Rul, 2000; Hebert et al., 2008; Kunji et al., 1996). Two additional cleavage sites were found at the $P_2$-$K_3$ and $E_{18}$-$N_{19}$ positions. The cleavage site $E_{18}$-$N_{19}$ has been already reported for the CEP of *Lb. delbrueckii* subsp. *lactis* CRL 581 (Hebert et al., 2008), whereas the cleavage site $P_2$-$K_3$ has never been identified in any CEPs previously described from lactobacilli. These results collectively suggested that CEPs from *Lb.*

*casei* PRA205 and *Lb. rhamnosus* PRA331 could belong to the mixed $P_I/P_{III}$-type. This result disagrees with the $P_I$-type CEP previously characterized in *Lb. casei* HN14 (Kojic et al., 1991), while it is consistent with the mixed $P_I/P_{III}$ type CEPs isolated from *Lb. rhamnosus* CGMCC11055 and *Lb. casei* subsp. *casei* IFLP 731 (Guo et al., 2016; Fernández de Palencia et al., 1997). Overall, these evidences strongly support the high level of intra- and inter-species variability in protease repertoire exhibited by lactobacilli (Liu, Bayjanov, Renckens, Nauta, & Siezen, 2010). As reported above, the cell viability near to 100% measured at the end of the fermentation trials allowed us to exclude that intracellular peptidase released from lysed cells may contribute to the hydrolysis of the fragment αS1-casein (f1-23). Indeed, as reported by Christensen, Broadbent, & Steele (2003), the presence of cytoplasmic peptidase should results in an almost complete breakdown of the peptide αS1-casein (f1-9).

*3.4. Analysis of the β-casein cleavage site-specificity*

The cleavage site-specificity of PRA205 and PRA331 CEPs was determined using β-casein as preferred substrate (**Figure 3**). In total, 76 and 72 different cleavage sites were detected in samples hydrolysed by PRA205 and PRA331 whole cells, respectively. They constitute 36.5 and 34.6% of all peptide bonds present in β-casein, showing that *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 CEPs have a very broad substrate specificity. These CEPs have almost the same specificity, as they shared 65% of cleavage sites.

Amino acid sequence analysis of the identified peptides revealed that the cleavage sites were not concentrated at the N- or C-terminus, but rather distributed throughout the entire β-casein sequence for both the CEPs activities (**Figure 3**). Most of the proteinases previously described in lactobacilli have been proven to preferentially hydrolyse the C-terminal of β-casein (Lozo et al., 2011). Recently, the PrtP proteinase isolated from *Lb. rhamnosus* CGMCC11055 breakdowns sites distributed along the whole β-casein sequence, like PRA205 and PRA331 CEPs (Guo et al., 2016).

Furthermore, we calculated the cleavage probability (%*Pn*) of the *Lb. casei* PRA205 CEP at the P1 and P1' positions **(Table 1)**. This CEP cleaved preferentially when the P1 position was occupied by the hydrophobic amino acids M, L and F or the negatively charged amino acids Q and N primarily, and by the polar un-charged amino acid E to a lesser extent. **Table 1** also shows how the amino acids at the P1' position affected cleavage occurrence. PRA205 CEP exhibited cleavage preference towards the residues S, N, A and H in this position, whereas had a reduced preference for M, D, R and Y. Coefficients *Kn* were calculated to quantify the influence of different amino acid residues on the P1-P1'cleavage probabilities (**Figure 4**). Amino acids N, M, Q, F and L in the P1 position and amino acids S, N, A and H in the P1' position exerted the strongest positive effects on cleavage occurrence. The amino acids E in P1 position and V, M, D, R and Y in P1' position also exerted a positive but weaker effect on cleavage probability. By contrast, G, I, P and D in the P1 position and P and I in the P1' position strongly inhibited the cleavage probability. Similarly, a negative effect was also found for the amino acids E and T at the P1' position. Previous works found that CEPs preferentially cleave negatively charged and hydrophobic amino acids (Hebert et al., 2008; Juillard et al., 1995; Lozo et al., 2011; Monnet, Ley, & Gonzalez, 1992). For instance, Q and E at the P1 position positively affect the cleavage by CEP from *Lb. delbrueckii* subsp. *lactis* CRL 581 (Hebert et al., 2008), whereas the occurrence of Q and F at the same position positively affects the cleavage by CEPs from *Lb. rhamnosus* BGT10, *Lb. helveticus* BGRA43 and *Lb. paracasei* subsp. *paracasei* BGHN14 (Lozo et al., 2011). In *Lb. casei* PRA205 CEP exhibited a pattern of cleavage site preferences similar to $P_I/P_{III}$-type CEP described in *Lc. lactis* subsp. *lactis* strain NCDO763 (Monnet, Ley, & Gonzalez, 1992), but different from those described for the $P_I$-type CEP in *Lc. lactis* subsp. *cremoris* strain Wg2 and for the $P_I/P_{III}$-type CEP in *Lb. rhamnosus* strain CGMCC11055. In strain Wg2 the residue Y at the P1 position and the residues N and T at the P1' position were preferred (Juillard et al., 1995), whereas in strain CGMCC11055 the residue P was preferred in both P1 and P1' subsites (Guo et al., 2016). By contrast, strain NCDO763 had a CEP activity positively affected by Q and N at the P1 position, and by S and A at the P1' position

(Monnet et al., 1992). Additionally, the residue P in both the P1 and P1' positons negatively affected CEP cleavage in NCDO763 (Monnet et al., 1992). Similarly, the presence of a P residue bound to one of the preferred cleaved amino acids prevented CEP from *Lb. casei* PRA205 to cut the peptidic bond. For example, the preferentially cleaved amino acids Q and L formed seven and nine peptidic bonds with the amino acid P, respectively, but no one of these bonds was cleaved by PRA205 CEP (**Figure 3**). Finally, PRA205 CEP activity displayed the following two unique properties: M at the P1 position exerted a strong positive effect on cleavage occurrence, whereas I in both the P1 and P1' positions exerted a strong negative effect. To the best of our knowledge, this cleavage site-specificity pattern has never been described in lactobacilli.

As reported in **Table 1** and **Figure 4**, CEP from *Lb. rhamnosus* PRA331 had a profile of cleavage specificity similar to *Lb. casei* PRA205. The main differences were the negative effect exerted by the amino acid H at the P1 position and the lack of the positive effect exerted by A at the P1' position.

*3.5. Identification of bioactive peptides using functional peptides databases*

The peptides cleaved by PRA205 and PRA331 CEPs in milk hydrolysates were searched against the general bioactive peptide database BIOPEP (Minkiewicz et al., 2008) and the milk bioactive peptide database MBPDB (Nielsen et al., 2017), in order to find peptides which match sequences to known bioactive peptides. Out of 331 identified peptides, 24 shared 100% homologies with functional peptides previously reported to have various bioactivities (**Table 2**). These bioactive peptides represented 13.5% and 13.7% of the peptides totally released by *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 whole cells, respectively. Twenty-one peptides were commonly released by both the strains, whereas three peptides were uniquely identified in samples hydrolysed by *Lb. casei* PRA205 (**Figure 1**). Nineteen bioactive peptides derived from β-casein, four from αS1-casein and one from αS2-casein, whereas no bioactive peptides were found from κ-casein. (**Table 2**). The three *Lb. casei* PRA205-specific bioactive peptides were the β-casein fragments 192-209

(LYQEPVLGPVRGPFPIIV), 58-72 (LVYPFPGPIPNSLPQ) and 8-14 (VPGEIVE). Eighteen peptides were ACE-inhibitors, two had immunomodulatory activity, one showed dipeptidyl-peptidase IV (DPPIV) inhibitory activity and one was an antimicrobial peptide. Two peptides, VYPFPGPIPN and YPFPGPIPN, were multi-functional bioactive peptides with ACEi, antioxidant and opioid agonist or ACEi, DPPIV-inhibitory and opioid agonist activities, respectively (**Table 2**). Among the peptides with ACEi activity, YPFPGPIPN, KVLPVPQ, RPKHPIKHQ and LHLPLP showed *in vivo* antihypertensive activity in spontaneously hypertensive rat (Maeno, Yamamoto, & Takano, 1996; Quirós et al., 2007; Saito, Nakamura, Kitazawa, Kawai, & Itoh, 2000). For all the other identified bioactive peptides, the bioactivity was previously demonstrated with *in vitro* assays.

The physiological effects of bioactive peptides depend on their capability to arrive at the target organs in an active form (Udenigwe, & Fogliano, 2017). This required resistance to gastrointestinal proteases and brush border membrane peptidases, and absorption through the intestinal epithelium. Usually, P-containing peptides are considered resistant to degradation by digestive proteases. Peptides containing from one to four P residues in their sequences and with, in many cases, P at or near to carboxylic end, were found to survive *in vitro* gastro-intestinal digestion (Tagliazucchi et al., 2016). Among the identified bioactive peptides, seven of them were able to survive *in vitro* gastro-intestinal digestion (Picariello et al., 2015; Tagliazucchi et al., 2016) and were also found in human gastro-intestinal tract (Boutrou et al., 2013), namely DKIHPF, VYPFPGPIPN, YPFPGPIPN, NIPPLTQTPV, LHLPLP, FVAPFPEVF and VAPFPEVF. The intestinal brush-border membrane and the colonic cells also contain aminopeptidases e specific prolyl peptidases. However, the great quantity of P-rich peptides and the presence of peptides with inhibitory activities (as for example against DPP-IV and intestinal ACE) may slow down the action of prolyl peptidases, protecting the short peptides from hydrolysis and favouring their biological actions. Short peptides (two or three amino acids) are absorbed intact across the brush border membrane by a specific peptide transport system, whereas largest peptides via paracellular and/or

transcellular mechanisms (Vermeirssen, Van Camp, & Verstraete, 2004). The peptide LHLPLP showed *in vivo* anti-hypertensive activity in rats, and, after incubation with Caco-2 cells, it was hydrolysed by cellular peptidases to HLPLP prior to transport across the intestinal epithelium (Quirós, Dávalos, Lasunción, Ramos, & Recio, 2008). The penta-peptide HLPLP showed an absolute bioavailability of 5.2% and an absorption half-life of 2.8 min in rats (Sánchez-Rivera et al., 2014). HLPLP was found to be hydrolysed by plasma peptidases in shorter peptides, which retained the anti-hypertensive properties in rats (Quirós et al., 2008; Sánchez-Rivera et al., 2014; Sánchez-Rivera et al., 2016). No data on absorption or pharmacokinetics of the other identified bioactive peptides are available in literature.

*3.6. Bioactive peptides abundance across PRA205 and PRA331 fermented milk*

Each identified bioactive peptide was relatively quantified in the samples by integrating the area under the peak (AUP) from the extracted ion chromatogram. The ionization of specific peptides in mass spectrometry experiments is a major limitation in quantitative analysis with electrospray ionization mass spectrometry (ESI-MS). The relative ionization of individual peptides is dependent on intrinsic and extrinsic factors. The most important extrinsic factor is the so-called "matrix effect" which is caused by the co-elution of matrix components (typically salts, ions, highly polar compounds and carbohydrates) that alter, either suppressing or enhancing, the ionization of the target analyte (Furey, Moriarty, Bane, Kinsella, & Lehane 2013). The intrinsic factor are related to the amino acid sequence of the peptides. Some amino acids, such as basic or hydrophobic amino acids, are ionized more efficiently than the others and gave more intense signal in ESI-MS experiments (Cech, & Enke, 2000). Here, we compared the relative amount (expressed as AUP) of the same peptide in two different hydrolysates coming from the same matrix (fermented milk), thus we can exclude errors related to the extrinsic effect and assume that differences in peak intensity of the same analyte accurately reflects relative differences in its abundance.

Among the bioactive peptides detected in both milk hydrolysates, 17 exhibited mean abundances significantly different between *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 ($P<0.05$). In particular, five peptides were more abundant in *Lb. casei* PRA205 milk hydrolysates and twelve in *Lb. rhamnosus* PRA331 milk hydrolysates (**Table 2**). When peptides intensities were summed, the bioactive peptides released by *Lb. rhamnosus* PRA331 whole cells were significantly higher than those released by *Lb. casei* PRA205 ($11.50 \times 10^{10} \pm 0.39 \times 10^{10}$ vs. $8.63 \times 10^{10} \pm 0.37 \times 10^{10}$; $P=0.0007$).

## 4. Conclusions

Nowadays, there is an increasing interest in developing novel dairy healthy products. Studies on strains *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 may represent a proof-of-concept of the working flowchart to develop novel functional adjunct culture and the subsequent functional delivery food. We isolated proteolytic and stress-resistant strains from a stressful food niche (no sugars available, high salt concentration, low aw), such as Parmigiano Reggiano, and identified them using a rigorous polyphasic identification frame-shift (Solieri et al., 2012). We demonstrated that all of them are safe (sensitive to all tested antibiotics) and some resistant to *in vitro* gastro-intestinal conditions (Solieri et al., 2014). We positively tested their ability to release VPP and IPP both in milk (Solieri et al., 2015) and in yogurt (Rutella et al., 2016), supporting the development of a double functional food, i.e. yogurt enriched in potentially probiotic viable cells and in antihypertensive peptides released by themselves. In this work, we characterized the CEPs that are the first enzymatic activities responsible for these relevant proteolytic features. For this purpose, a cutting-edge peptidomic approach was implemented in order to define the pattern of caseins breakdown by CEP activity from whole cells grown in milk. We demonstrated that CEPs activities of *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331showed two unique features: a new cleavage site ($P_2$-$K_3$) on the αS1-casein fragment 1-23 and a novel pattern of β-casein cleavage site-specificity. Through a BIOPEP and MBPDB databases analysis, we also demonstrated that several identified

peptides matched the sequences of previously reported bioactive peptides. This information could be relevant, mainly considering the wide heterogeneity in distribution of different proteinase-encoding genes among and within *Lactobacillus* species. Comparative genome analysis showed that lactobacilli strongly differ in the components of their proteolytic systems at strain level (Liu et al., 2010). This strain-specificity accounts for the high phenotypic diversity in caseinolytic activity and in of the resulting released bioactive peptides, as well as makes necessary to deeply characterize each strain selected for functional food applications. However, it is important to note that protein hydrolysis catalysed by proteases is a dynamic process and that, in the present work, bioactive peptides were identified in fermented milk samples at one single time. We cannot exclude that shorter or longer incubation of milk with *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 whole cells may result in a different bioactive peptide profile of the samples. In addition, future biochemical assays with the synthesized peptides are needed to complement the *in silico* evidences collected here.

Overall, the results provided in the present work will increase the knowledge about the proteolytic system of two important NS-LAB species, such as *Lb. casei* and *Lb. rhamnosus*, which are poorly studied compared to the best-described lactococci and thermophilic lactobacilli. Finally, since strains PRA205 and PRA331 released several potential bioactive peptides, they could be promising functional starters or adjunct cultures for formulating dairy products with health properties.

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**Figure Captions**

**Figure 1. Venn diagram showing differences between *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 CEPs in patterns of peptides and bioactive peptides cleaved from milk caseins.** The complete pattern of peptides identified at the end of the fermentation trials by mass spectrometry can be found in Supplementary on line **Tables S1-S8**. In the preparation of the Venn diagram related to bioactive peptides, only peptides found from the literature to have 100% homology to known functional peptides were reported in the **Figure**. Peptides present in at least two of a triplicate's samples were considered present.

**Figure 2**. **Specificity of CEPs from strains *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 toward αS1-casein fragment 1-23**. The cleavage sites are indicated by arrows.

**Figure 3. Distribution of the cleavage sites identified in the primary sequences of β-casein by CEPs from *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331.** The cleavage sites are indicated by arrows.

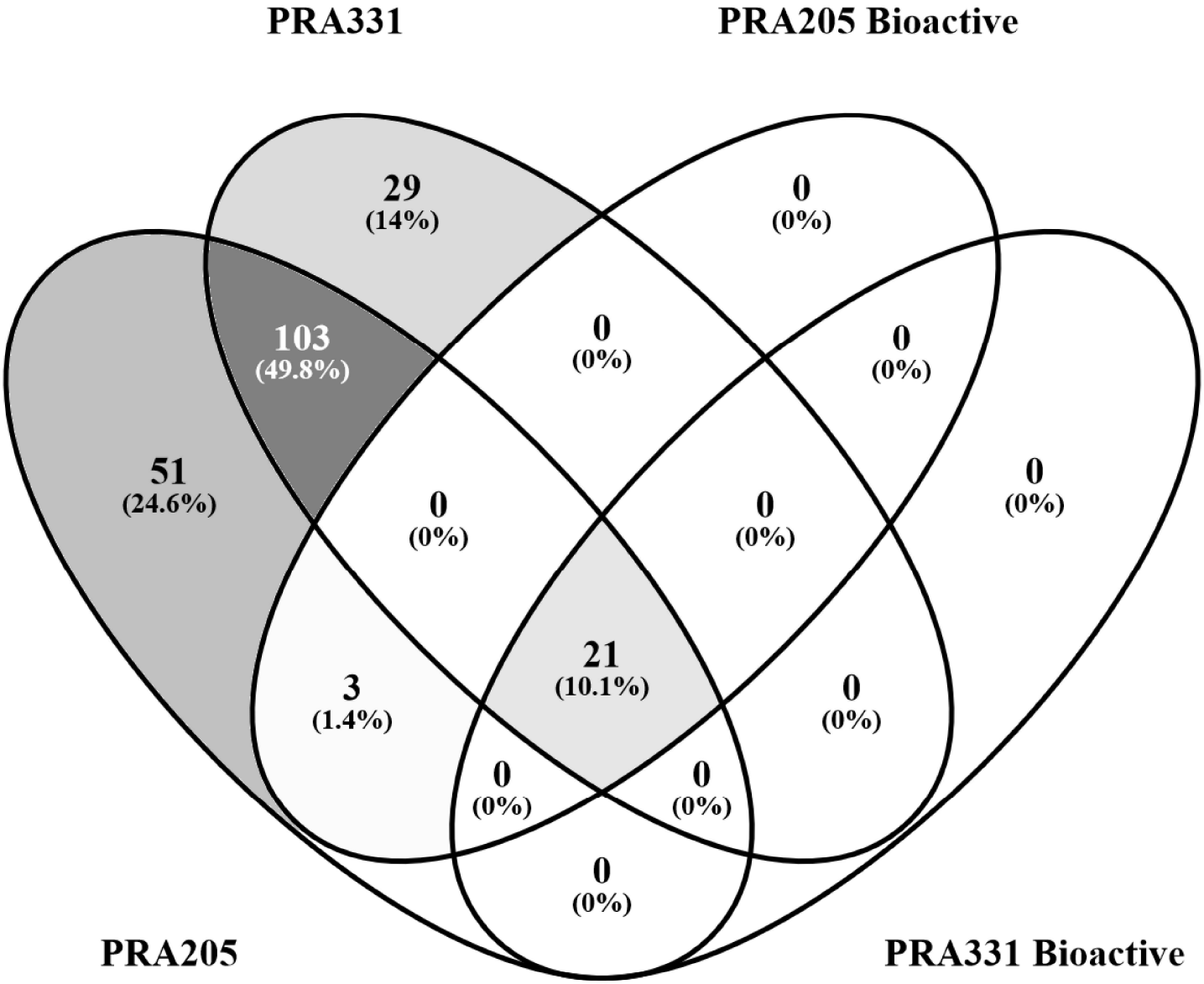
**Figure 4**. **Cleavage preference of *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 CEPs towards eighteen amino acids at the P1 and P1' subsites.** (**A**) Influence of the different amino acids in the P1 subsite by CEP from *Lb. casei* PRA205. (**B**) Influence of the different amino acids in the P1' subsite by CEP from *Lb. casei* PRA205. (**C**) Influence of the different amino acids in the P1 subsite by CEP from *Lb. rhamnosus* PRA331. (**D**) Influence of the different amino acids in the P1' subsite by CEP from *Lb. rhamnosus* PRA331. See materials and methods section for the calculation of the coefficient *Kn*. Positive and negative values indicate a positive or negative influence exerted by each residue on the cleavage of the P1-P1' bond, respectively. Please note that the amino acid C is not present in the sequence of β-casein, whereas W was omitted from the analysis since it occurs once in the β-casein sequence.

**Table 3**. Peptides with previously demonstrated bioactivity identified in the milk hydrolysates by whole cells of *Lactobacillus casei* PRA205 or *Lactobacillus rhamnosus* PRA331.

| *Sequence* | *Fragment* | *Bioactivity* | *PRA205 relative amount[a] (±SD)* | *PRA331 relative amount[a] (±SD)* | *P-value* | *Reference* |
|---|---|---|---|---|---|---|
| LNVPGEIVE | β-casein f(6-14) | ACEi | $1.13x10^9 \pm 1.26x10^8$ | $1.30x10^9 \pm 4.21x10^7$ | 0.0876 | Gobbetti et al., 2000 |
| VPGEIVE | β-casein f(8-14) | DPPIV-inhibitor | $4.30x10^8 \pm 3.38x10^7$ | n.d. | / | Nongonierma et al., 2016 |
| DKIHPF | β-casein f(47-52) | ACEi | $2.37x10^{10} \pm 3.37x10^9$ | $2.36x10^{10} \pm 6.25x10^8$ | 0.4925 | Gobbetti et al., 2000 |
| LVYPFPGPIPNSLPQ | β-casein f(58-72) | ACE-inhibitor | $3.44x10^8 \pm 3.89x10^7$ | n.d. | / | Smacchi et al., 2008 |
| VYPFPGPIPN | β-casein f(59-68) | ACEi<br>Antioxidant<br>Opioid agonist | $8.19x10^9 \pm 7.04x10^8$ | $1.62x10^{10} \pm 4.17x10^8$ | 0.0004 | Eisele et al., 2013 |
| YPFPGPIPN | β-casein f(60-68) | ACEi<br>DPPIV-inhibitor<br>Opioid agonist | $3.34x10^9 \pm 1.30x10^8$ | $1.03x10^{10} \pm 1.89x10^9$ | 0.0030 | Saito et al., 2000 |
| NIPPLTQTPV | β-casein f(73-82) | ACEi | $9.51x10^9 \pm 5.72x10^8$ | $5.96x10^9 \pm 4.64x10^8$ | 0.0027 | Gobbetti et al., 2000 |
| NLHLPLP | β-casein f(132-138) | ACEi | $1.94x10^9 \pm 2.09x10^8$ | $7.81x10^8 \pm 1.22x10^8$ | 0.0031 | Kohmura et al., 1989 |
| NLHLPLPLL | β-casein f(132-140) | ACEi | $8.78x10^8 \pm 3.33x10^7$ | $5.92x10^8 \pm 1.39x10^7$ | 0.0037 | Robert et al., 2004 |
| LHLPLP | β-casein f(133-138) | ACEi | $3.40x10^8 \pm 3.47x10^7$ | $7.64x10^8 \pm 6.41x10^7$ | 0.0072 | Kohmura et al., 1989 |
| LHLPLPL | β-casein f(133-139) | ACEi | $2.55x10^9 \pm 2.85x10^8$ | $3.06x10^9 \pm 2.19x10^8$ | 0.0920 | Quiros et al., 2007 |
| SQSKVLPVPQ | β-casein f(166-175) | ACEi | $3.49x10^8 \pm 3.51x10^7$ | $6.14x10^8 \pm 7.03x10^7$ | 0.0050 | Hayes et al., 2007 |
| SKVLPVPQ | β-casein f(168-175) | ACEi | $8.32x10^8 \pm 1.07x10^8$ | $1.07x10^9 \pm 7.70x10^7$ | 0.0380 | Yamamoto et al., 1994 |
| KVLPVPQ | β-casein f(169-175) | ACEi | $6.05x10^9 \pm 5.74x10^8$ | $1.40x10^{10} \pm 1.76x10^9$ | 0.0022 | Maeno et al., 1996 |
| RDMPIQAF | β-casein f(183-190) | ACEi | $7.64x10^9 \pm 2.57x10^8$ | $1.33x10^9 \pm 8.06x10^7$ | <0.0001 | Yamamoto et al., 1994 |
| LYQEPVLGPVRGPFPIIV | β-casein f(192-209) | Immunomodulator | $3.19x10^8 \pm 4.13x10^7$ | n.d. | | Boutrou et al., 2013 |
| YQEPVLGPVRGPFPIIV | β-casein f(193-209) | Immunomodulator | $1.69x10^9 \pm 3.86x10^8$ | $1.21x10^9 \pm 1.57x10^8$ | 0.1237 | Boutrou et al., 2013 |
| QEPVLGPVRGPFPIIV | β-casein f(194-209) | ACEi | $7.53x10^9 \pm 1.96x10^8$ | $1.16x10^{10} \pm 5.14x10^8$ | 0.0044 | Lu et al., 2016 |
| EPVLGPVRGPFP | β-casein f(195-206) | ACEi | $3.79x10^8 \pm 3.38x10^7$ | $4.90x10^8 \pm 4.07x10^7$ | 0.0219 | Hayes et al., 2007 |
| RPKHPIKHQ | αS1-casein f(1-9) | ACEi | $7.10x10^9 \pm 6.63x10^8$ | $1.73x10^{10} \pm 2.66x10^9$ | 0.0032 | Saito et al., 2000 |
| ENLLRF | αS1-casein f(18-24) | ACEi | $8.23x10^8 \pm 1.76x10^8$ | $1.46x10^9 \pm 2.66x10^8$ | 0.0226 | Boutrou et al., 2013 |

| | | | | | | |
|---|---|---|---|---|---|---|
| FVAPFPEVF | αS1-casein f(24-32) | ACEi | $7.27x10^8 \pm 1.16x10^8$ | $1.80x10^9 \pm 6.93x10^7$ | 0.0039 | Boutrou et al., 2013 |
| VAPFPEVF | αS1-casein f(25-32) | ACEi | $3.17x10^8 \pm 3.39x10^7$ | $1.48x10^9 \pm 1.37x10^8$ | 0.0003 | Boutrou et al., 2013 |
| TKVIPYVRYL | αS2-casein f(198-207) | Antimicrobial | $1.62x10^8 \pm 3.75x10^7$ | $5.30x10^7 \pm 2.11x10^7$ | 0.0180 | Alvarez-Ordóñez et al., 2013 |

Abbreviations are: ACEi, angiotensin converting enzyme-inhibitory; DPPIV, dipeptidyl peptidase IV.
[a]Amounts were calculated by measuring the area under the peak (AUP) from the extracted ion chromatograms (EIC) obtained for each peptide and AUP values were normalized to the total peptide content of the milk hydrolysates. Values are means ± standard deviation. Statistically significant differences between PRA205 and PRA331 samples were calculated by Student's t-test ($P<0.05$).


PRA331
PRA205 Bioactive
29
(14%)
0
(0%)
103
(49.8%)
0
(0%)
0
(0%)
51
(24.6%)
0
(0%)
0
(0%)
0
(0%)
21
(10.1%)
3
(1.4%)
0
(0%)
0
(0%)
0
(0%)
0
(0%)
PRA205
PRA331 Bioactive

| | RPKHPIKHQGLPQEVLNENLLRF |
|---|---|
| PRA205 | ↑ ↑ ↑ ↑ ↑ ↑ ↑ |
| PRA331 | ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ |

RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEE

PRA205 ↑↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑↑

PRA331 ↑↑ ↑ ↑ ↑ ↑

QQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQ

PRA205 ↑ ↑ ↑ ↑↑↑↑ ↑ ↑

PRA331 ↑ ↑ ↑ ↑↑↑↑ ↑ ↑ ↑

NIPPLTQTPVVVPPFLQPEVMGVSKVKEAMAPKH

PRA205 ↑↑ ↑ ↑ ↑↑ ↑ ↑ ↑ ↑ ↑ ↑↑ ↑ ↑↑

PRA331 ↑↑ ↑ ↑ ↑ ↑↑ ↑ ↑ ↑ ↑ ↑ ↑↑ ↑↑

KEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQ

PRA205 ↑↑ ↑↑ ↑ ↑↑↑↑ ↑↑↑↑

PRA331 ↑↑ ↑↑ ↑ ↑↑↑↑ ↑↑↑↑

SWMHQPHQPLPPTVMFPPQSVLSLSQSKVLPVP

PRA205 ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑↑↑↑↑

PRA331 ↑ ↑ ↑ ↑ ↑ ↑ ↑ ↑↑↑↑↑

QKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV

PRA205 ↑ ↑ ↑ ↑↑↑↑↑↑ ↑ ↑↑↑ ↑ ↑ ↑

PRA331 ↑ ↑ ↑ ↑ ↑↑↑↑↑ ↑ ↑↑↑ ↑ ↑ ↑

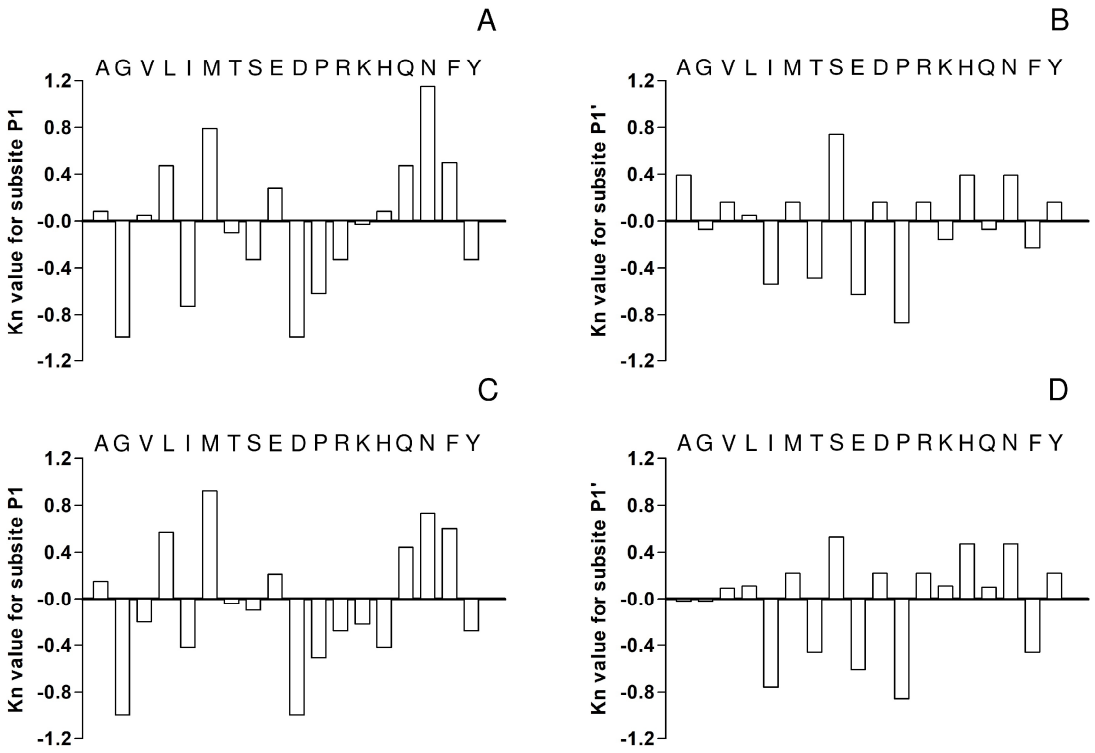

A
B
C
D
A G V L I M T S E D P R K H Q N F Y
Kn value for subsite P1
Kn value for subsite P1'
1.2
0.8
0.4
-0.0
-0.4
-0.8
-1.2

**Table 1.** Cleavage occurrence and cleavage probability (*%P*) produced by *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331 cell-envelope proteinase on β-casein at different amino acids in the P1 and P1'subsites.

| | *Lb. casei* PRA205 | | | *Lb. rhamnosus* PRA331 | | |
|---|---|---|---|---|---|---|
| **Amino acids**[a] | **Number of residues** | **P1 subsite** Number of cleaved bond[b] (%P1[c]) | **P1' subsite** Number of cleaved bond[b] (%P1'[c]) | **Number of residues** | **P1 subsite** Number of cleaved bond[b] (%P1[c]) | **P1' subsite** Number of cleaved bond[b] (%P1'[c]) |
| *Aliphatic amino acids* | | | | | | |
| A | 5 | 2 (40.0) | 3 (60.0) | 5 | 2 (40.0) | 2 (40.0) |
| G | 5 | 0 (0) | 2 (40.0) | 5 | 0 (0) | 2 (40.0) |
| V | 18 | 7 (38.9) | 9 (50.0) | 18 | 5 (27.8) | 8 (44.4) |
| L | 22 | 12 (54.6) | 10 (45.5) | 22 | 12 (54.6) | 10 (45.5) |
| I | 10 | 1 (10.0) | 2 (20.0) | 10 | 2 (20.0) | 1 (10.0) |
| M | 6 | 4 (66.7) | 3 (50.0) | 6 | 4 (66.7) | 3 (50.0) |
| *Polar un-charged amino acids* | | | | | | |
| T | 9 | 3 (33.3) | 2 (22.2) | 9 | 3 (33.3) | 2 (22.2) |
| S | 16 | 4 (25.0) | 12 (75.0) | 16 | 5 (31.3) | 10 (61.5) |
| E | 19 | 9 (47.4) | 3 (15.8) | 19 | 8 (42.1) | 3 (15.8) |
| D | 4 | 0 (0) | 2 (50.0) | 4 | 0 (0) | 2 (50.0) |
| P | 35 | 5 (14.3) | 2 (5.7) | 35 | 6 (17.1) | 2 (5.7) |
| *Positively charged amino acids* | | | | | | |
| R | 4 | 1 (25.0) | 2 (50.0) | 4 | 1 (25.0) | 2 (50.0) |
| K | 11 | 4 (36.4) | 4 (36.4) | 11 | 3 (27.3) | 5 (45.5) |
| H | 5 | 2 (40.0) | 3 (60.0) | 5 | 1 (20.0) | 3 (60.0) |
| *Negatively charged amino acids* | | | | | | |
| Q | 20 | 11 (55.0) | 8 (40.0) | 20 | 10 (50.0) | 9 (45.0) |
| N | 5 | 4 (80.0) | 3 (60.0) | 5 | 3 (60.0) | 3 (60.0) |
| *Aromatic amino acids* | | | | | | |
| F | 9 | 5 (55.6) | 3 (33.3) | 9 | 5 (55.7) | 2 (22.2) |
| Y | 4 | 1 (25.0) | 2 (50.0) | 4 | 1 (25.0) | 2 (50.0) |

[a]One code letter was used for amino acid nomenclature. The amino acid C is not present in the sequence of β-casein, while the amino acid W was omitted from the analysis since it occurs once in the β-casein sequence.
[b]The cleaved bonds are reported in **Figure 3**.
[c]See materials and methods section for the calculation of the *%P1* and *%P1'* cleavage probability.

**Table 2**. Peptides with previously demonstrated bioactivity identified in the milk hydrolysates by whole cells of *Lactobacillus casei* PRA205 or *Lactobacillus rhamnosus* PRA331.

| *Sequence* | *Fragment* | *Bioactivity* | *PRA205 relative amount[a] (±SD)* | *PRA331 relative amount[a] (±SD)* | *P-value* | *Reference* |
|---|---|---|---|---|---|---|
| LNVPGEIVE | β-casein f(6-14) | ACEi | $1.13x10^9 \pm 1.26x10^8$ | $1.30x10^9 \pm 4.21x10^7$ | 0.0876 | Gobbetti et al., 2000 |
| VPGEIVE | β-casein f(8-14) | DPPIV-inhibitor | $4.30x10^8 \pm 3.38x10^7$ | n.d. | / | Nongonierma et al., 2016 |
| DKIHPF | β-casein f(47-52) | ACEi | $2.37x10^{10} \pm 3.37x10^9$ | $2.36x10^{10} \pm 6.25x10^8$ | 0.4925 | Gobbetti et al., 2000 |
| LVYPFPGPIPNSLPQ | β-casein f(58-72) | ACE-inhibitor | $3.44x10^8 \pm 3.89x10^7$ | n.d. | / | Smacchi et al., 2008 |
| VYPFPGPIPN | β-casein f(59-68) | ACEi<br>Antioxidant<br>Opioid agonist | $8.19x10^9 \pm 7.04x10^8$ | $1.62x10^{10} \pm 4.17x10^8$ | 0.0004 | Eisele et al., 2013 |
| YPFPGPIPN | β-casein f(60-68) | ACEi<br>DPPIV-inhibitor<br>Opioid agonist | $3.34x10^9 \pm 1.30x10^8$ | $1.03x10^{10} \pm 1.89x10^9$ | 0.0030 | Saito et al., 2000 |
| NIPPLTQTPV | β-casein f(73-82) | ACEi | $9.51x10^9 \pm 5.72x10^8$ | $5.96x10^9 \pm 4.64x10^8$ | 0.0027 | Gobbetti et al., 2000 |
| NLHLPLP | β-casein f(132-138) | ACEi | $1.94x10^9 \pm 2.09x10^8$ | $7.81x10^8 \pm 1.22x10^8$ | 0.0031 | Kohmura et al., 1989 |
| NLHLPLPLL | β-casein f(132-140) | ACEi | $8.78x10^8 \pm 3.33x10^7$ | $5.92x10^8 \pm 1.39x10^7$ | 0.0037 | Robert et al., 2004 |
| LHLPLP | β-casein f(133-138) | ACEi | $3.40x10^8 \pm 3.47x10^7$ | $7.64x10^8 \pm 6.41x10^7$ | 0.0072 | Kohmura et al., 1989 |
| LHLPLPL | β-casein f(133-139) | ACEi | $2.55x10^9 \pm 2.85x10^8$ | $3.06x10^9 \pm 2.19x10^8$ | 0.0920 | Quiros et al., 2007 |
| SQSKVLPVPQ | β-casein f(166-175) | ACEi | $3.49x10^8 \pm 3.51x10^7$ | $6.14x10^8 \pm 7.03x10^7$ | 0.0050 | Hayes et al., 2007 |
| SKVLPVPQ | β-casein f(168-175) | ACEi | $8.32x10^8 \pm 1.07x10^8$ | $1.07x10^9 \pm 7.70x10^7$ | 0.0380 | Yamamoto et al., 1994 |
| KVLPVPQ | β-casein f(169-175) | ACEi | $6.05x10^9 \pm 5.74x10^8$ | $1.40x10^{10} \pm 1.76x10^9$ | 0.0022 | Maeno et al., 1996 |
| RDMPIQAF | β-casein f(183-190) | ACEi | $7.64x10^9 \pm 2.57x10^8$ | $1.33x10^9 \pm 8.06x10^7$ | <0.0001 | Yamamoto et al., 1994 |
| LYQEPVLGPVRGPFPIIV | β-casein f(192-209) | Immunomodulator | $3.19x10^8 \pm 4.13x10^7$ | n.d. | | Boutrou et al., 2013 |
| YQEPVLGPVRGPFPIIV | β-casein f(193-209) | Immunomodulator | $1.69x10^9 \pm 3.86x10^8$ | $1.21x10^9 \pm 1.57x10^8$ | 0.1237 | Boutrou et al., 2013 |
| QEPVLGPVRGPFPIIV | β-casein f(194-209) | ACEi | $7.53x10^9 \pm 1.96x10^8$ | $1.16x10^{10} \pm 5.14x10^8$ | 0.0044 | Lu et al., 2016 |
| EPVLGPVRGPFP | β-casein f(195-206) | ACEi | $3.79x10^8 \pm 3.38x10^7$ | $4.90x10^8 \pm 4.07x10^7$ | 0.0219 | Hayes et al., 2007 |
| RPKHPIKHQ | αS1-casein f(1-9) | ACEi | $7.10x10^9 \pm 6.63x10^8$ | $1.73x10^{10} \pm 2.66x10^9$ | 0.0032 | Saito et al., 2000 |
| ENLLRF | αS1-casein f(18-24) | ACEi | $8.23x10^8 \pm 1.76x10^8$ | $1.46x10^9 \pm 2.66x10^8$ | 0.0226 | Boutrou et al., 2013 |

| | | | | | | |
|---|---|---|---|---|---|---|
| FVAPFPEVF | αS1-casein f(24-32) | ACEi | $7.27x10^8 \pm 1.16x10^8$ | $1.80x10^9 \pm 6.93x10^7$ | 0.0039 | Boutrou et al., 2013 |
| VAPFPEVF | αS1-casein f(25-32) | ACEi | $3.17x10^8 \pm 3.39x10^7$ | $1.48x10^9 \pm 1.37x10^8$ | 0.0003 | Boutrou et al., 2013 |
| TKVIPYVRYL | αS2-casein f(198-207) | Antimicrobial | $1.62x10^8 \pm 3.75x10^7$ | $5.30x10^7 \pm 2.11x10^7$ | 0.0180 | Alvarez-Ordóñez et al., 2013 |

Abbreviations are: ACEi, angiotensin converting enzyme-inhibitory; DPPIV, dipeptidyl peptidase IV. Only peptides found from the literature to have 100% homology to known functional peptides were reported in the **Table**. The complete list of identified peptides can be found in Supplementary on line **Tables S1-S8**.

[a]Amounts were calculated by measuring the area under the peak (AUP) from the extracted ion chromatograms (EIC) obtained for each peptide and AUP values were normalized to the total peptide content of the milk hydrolysates as described in section 2.6. Values are means ± standard deviation. Statistically significant differences between PRA205 and PRA331 samples were calculated by Student's t-test ($P<0.05$).

**Figure S1**. β-casein derived peptides identified in the TCA-soluble supernatant obtained from milk fermented with *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. The peptides are reported as bars below the corresponding amino acid sequences. Red bars correspond to peptides with angiotensin-converting enzyme (ACE) inhibitory activity. Yellow bars correspond to peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. Green bars correspond to peptides with antioxidant activity. Light blue bars correspond to peptides with anti-microbial activity. Blue bars correspond to peptides with opioid agonist activity. Purple bars correspond to peptides with immunomodulation activity. Peptides with more than one activity are presented with multi-colour bars.

**Figure S2**. αS1-casein derived peptides identified in the TCA-soluble supernatant obtained from milk fermented with *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. The peptides are reported as bars below the corresponding amino acid sequences. Red bars correspond to peptides with angiotensin-converting enzyme (ACE) inhibitory activity. Yellow bars correspond to peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. Green bars correspond to peptides with antioxidant activity. Light blue bars correspond to peptides with anti-microbial activity. Blue bars correspond to peptides with opioid agonist activity. Purple bars correspond to peptides with immunomodulation activity. Peptides with more than one activity are presented with multi-colour bars.

**Figure S3**. αS2-casein derived peptides identified in the TCA-soluble supernatant obtained from milk fermented with *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. The peptides are reported as bars below the corresponding amino acid sequences. Red bars correspond to peptides with angiotensin-converting enzyme (ACE) inhibitory activity. Yellow bars correspond to peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. Green bars correspond to peptides with antioxidant activity. Light blue bars correspond to peptides with anti-microbial activity. Blue bars correspond to peptides with opioid agonist activity. Purple bars correspond to

peptides with immunomodulation activity. Peptides with more than one activity are presented with multi-colour bars.

**Figure S4**. κ-casein derived peptides identified in the TCA-soluble supernatant obtained from milk fermented with *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. The peptides are reported as bars below the corresponding amino acid sequences. Red bars correspond to peptides with angiotensin-converting enzyme (ACE) inhibitory activity. Yellow bars correspond to peptides with dipeptidyl peptidase IV (DPP-IV) inhibitory activity. Green bars correspond to peptides with antioxidant activity. Light blue bars correspond to peptides with anti-microbial activity. Blue bars correspond to peptides with opioid agonist activity. Purple bars correspond to peptides with immunomodulation activity. Peptides with more than one activity are presented with multi-colour bars.

*Lactobacillus casei* PRA205

RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQNIPP

LTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQ

PHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV

*Lactobacillus rhamnosus* PRA331

RELEELNVPGEIVESLSSSEESITRINKKIEKFQSEEQQQTEDELQDKIHPFAQTQSLVYPFPGPIPNSLPQNIPP

LTQTPVVVPPFLQPEVMGVSKVKEAMAPKHKEMPFPKYPVEPFTESQSLTLTDVENLHLPLPLLQSWMHQ

PHQPLPPTVMFPPQSVLSLSQSKVLPVPQKAVPYPQRDMPIQAFLLYQEPVLGPVRGPFPIIV

*Lactobacillus casei* PRA205

RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQMEAESISSSEEIVPNSV

EQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFR

QFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW

*Lactobacillus rhamnosus* PRA331

RPKHPIKHQGLPQEVLNENLLRFFVAPFPEVFGKEKVNELSKDIGSESTEDQAMEDIKQMEAESISSSEEIVPNSV

EQKHIQKEDVPSERYLGYLEQLLRLKKYKVPQLEIVPNSAEERLHSMKEGIHAQQKEPMIGVNQELAYFYPELFR

QFYQLDAYPSGAWYYVPLGTQYTDAPSFSDIPNPIGSENSEKTTMPLW

*Lactobacillus casei* PRA205

KNTMEHVSSSEESIISQETYKQEKNMAINPSKENLCSTFCKEVVRNANEEEYSIGSSSEESAEVATEEVKITVDDK

HYQKALNEINQFYQKFPQYLQYLYQGPIVLNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVFTKK

TKLTEEEKNRLNFLKKISQRYQKFALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL

*Lactobacillus rhamnosus* PRA331

KNTMEHVSSSEESIISQETYKQEKNMAINPSKENLCSTFCKEVVRNANEEEYSIGSSSEESAEVATEEVKITVDDK

HYQKALNEINQFYQKFPQYLQYLYQGPIVLNPWDQVKRNAVPITPTLNREQLSTSEENSKKTVDMESTEVFTKK

TKLTEEEKNRLNFLKKISQRYQKFALPQYLKTVYQHQKAMKPWIQPKTKVIPYVRYL

*Lactobacillus casei* PRA205

QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQ

WQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDS

PEVIESPPEINTVQVTSTAV

*Lactobacillus rhamnosus* PRA331

QEQNQEQPIRCEKDERFFSDKIAKYIPIQYVLSRYPSYGLNYYQQKPVALINNQFLPYPYYAKPAAVRSPAQILQ

WQVLSNTVPAKSCQAQPTTMARHPHPHLSFMAIPPKKNQDKTEIPTINTIASGEPTSTPTTEAVESTVATLEDS

PEVIESPPEINTVQVTSTAV

**Table S1**. β-casein-derived peptides identified in milk fermented with *Lactobacillus casei* PRA205 whole cells[a].

| *Sequence*[b] | *Observed mass (m/z)*[c] | *Calculated mass*[d] | *Fragment* | *Bioactivity*[e] | *Reference* |
|---|---|---|---|---|---|
| LNVPGEIVE | 969.5562 | 968.5179 | f(6-14) | ACEi | Gobbetti et al., 2000 |
| NVPGEIVE | 856.4430 | 855.4338 | f(7-14) | / | / |
| VPGEIVE | 742.3943 | 741.3909 | f(8-14) | DPPIV-inhibitor | Nongonierma et al., 2016 |
| SITRIN | 352.1970 | 702.4024 | f(22-27) | / | / |
| KKIEKF | 396.7429 | 791.4905 | f(28-33) | / | / |
| KKIEKFQ | 460.7709 | 919.5491 | f(28-34) | / | / |
| KKIEKFQS(phospho)E | 608.8120 | 1215.5900 | f(28-36) | / | / |
| KKIEKFQS(phospho)EE | 673.3181 | 1344.6326 | f(28-37) | / | / |
| IEKFQS(phospho)EE | 545.2067 | 1088.4427 | f(30-37) | / | / |
| DKIHPF | 756.3786 | 755.3966 | f(47-52) | ACEi | Gobbetti et al., 2000 |
| DKIHPFAQTQ | 592.7785 | 1183.5986 | f(47-56) | / | / |
| SLVYPFPGPIPN | 650.8484 | 1299.6863 | f(57-68) | / | / |
| SLVYPFPGPIPNSLPQ | 863.4501 | 1724.9138 | f(57-72) | / | / |
| LVYPFPGPIPN | 1213.6762 | 1212.6543 | f(58-68) | / | / |
| LVYPFPGPIPNSLPQ | 819.9604 | 1724.9138 | f(58-72) | ACEi | Smacchi et al., 2008 |
| VYPFPGPIPN | 1100.5568 | 1099.5702 | f(59-68) | ACEi<br>Antioxidant<br>Opioid agonist | Eisele et al., 2013 |
| VYPFPGPIPNSLPQ | 763.4073 | 1524.7977 | f(59-72) | / | / |
| YPFPGPIPN | 1001.5291 | 1000.5018 | f(60-68) | ACEi<br>DPPIV-inhibitor<br>Opioid agonist | Saito et al., 2000 |
| SLPQNIPPL | 978.5771 | 977.5546 | f(69-77) | / | / |
| SLPQNIPPLTQTPVVVPPFLQPEVM(ox) | 919.8153 | 2755.4823 | f(69-93) | / | / |
| NIPPLTQTPV | 1079.6176 | 1078.6023 | f(73-82) | ACEi | Gobbetti et al., 2000 |

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|---|---|---|---|---|---|
| NIPPLTQTPVVVPPF | 809.9563 | 1617.9131 | f(73-87) | / | / |
| NIPPLTQTPVVVPPFLQPEVM | 772.7640 | 2315.2599 | f(73-93) | / | / |
| TQTPV | 545.2884 | 544.2857 | f(78-82) | / | / |
| TQTPVVVPPF | 1084.5754 | 1083.5965 | f(78-87) | / | / |
| TQTPVVVPPFLQPE | 776.4502 | 1550.8345 | f(78-91) | / | / |
| TQTPVVVPPFLQPEVM | 891.4896 | 1780.9434 | f(78-93) | / | / |
| TQTPVVVPPFLQPEVMGV | 969.5306 | 1937.0333 | f(78-95) | / | / |
| TQTPVVVPPFLQPEVMGVSKVKEAMAP | 960.5079 | 2878.5337 | f(78-104) | / | / |
| QTPVVVPPFLQPEVM | 840.9568 | 1679.8957 | f(79-93) | / | / |
| PVVVPPFLQPEVM | 726.4071 | 1450.7894 | f(81-93) | / | / |
| VVPPFLQPE | 1025.6040 | 1024.5593 | f(83-91) | / | / |
| VVPPFLQPEVM | 1255.6987 | 1254.6682 | f(83-93) | / | / |
| VPPFLQPE | 463.7369 | 925.4909 | f(84-91) | / | / |
| VPPFLQPEVM | 578.7970 | 1155.5968 | f(84-93) | / | / |
| PPFLQPE | 827.4361 | 826.4225 | f(85-91) | / | / |
| LQPEVM | 716.3580 | 715.3575 | f(88-93) | / | / |
| AMAPKHKEMPFPKYPVEPF | 748.7340 | 2243.1271 | f(101-119) | / | / |
| MAPKHKEMPFPKYPVEPF | 725.0321 | 2172.0900 | f(102-119) | / | / |
| APKHKEMPFPKYPVEPF | 681.3579 | 2041.0495 | f(103-119) | / | / |
| APKHKEMPFPKYPVEPFTESQ | 622.5635 | 2486.2304 | f(103-123) | / | / |
| KHKEMPFPKYPVEPF | 625.3218 | 1872.9596 | f(105-119) | / | / |
| HKEMPFPKYPVEPF | 582.6372 | 1744.8647 | f(106-119) | / | / |
| HKEMPFPKYPVEPFTESQ | 730.9973 | 2190.0456 | f(106-123) | / | / |
| EMPFPKYPVEP | 667.3054 | 1332.6424 | f(108-118) | / | / |
| EMPFPKYPVEPF | 740.8620 | 1479.7108 | f(108-119) | / | / |
| MPFPKYPVEP | 602.8135 | 1203.5998 | f(109-118) | / | / |

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|---|---|---|---|---|---|
| MPFPKYPVEPF | 676.3415 | 1350.6682 | f(109-119) | / | / |
| NLHLPLP | 402.2601 | 801.4701 | f(132-138) | ACEi | Kohmura et al., 1989 |
| NLHLPLPL | 916.5616 | 915.5542 | f(132-139) | / | / |
| NLHLPLPLL | 515.3168 | 1028.6382 | f(132-140) | ACEi | Robert et al., 2004 |
| NLHLPLPLLQ | 579.3496 | 1156.6968 | f(132-141) | / | / |
| NLHLPLPLLQS | 622.8851 | 1243.7288 | f(132-142) | / | / |
| NLHLPLPLLQSW | 715.9295 | 1429.8082 | f(132-143) | / | / |
| LHLPLP | 345.2209 | 688.4272 | f(133-138) | ACEi | Kohmura et al., 1989 |
| LHLPLPL | 401.7546 | 801.5112 | f(133-139) | ACEi | Quiros et al., 2007 |
| LHLPLPLLQ | 1043.6493 | 1042.6539 | f(133-141) | / | / |
| LHLPLPLLQS | 565.8369 | 1129.6859 | f(133-142) | / | / |
| LHLPLPLLQSW | 658.9507 | 1315.7652 | f(133-143) | / | / |
| HLPLPLLQ | 465.7873 | 929.5698 | f(134-141) | / | / |
| HLPLPLLQSW | 602.3328 | 1202.6812 | f(134-143) | / | / |
| LPLPLLQ | 793.5148 | 792.5109 | f(135-141) | / | / |
| LPLPLLQSW | 1066.6182 | 1065.6223 | f(135-143) | / | / |
| WMHQPHQPLPPT | 490.2347 | 1467.7081 | f(143-154) | / | / |
| WMHQPHQPLPPTVM | 566.9374 | 1697.8170 | f(143-156) | / | / |
| MHQPHQPLPPT | 641.8116 | 1281.6288 | f(144-154) | / | / |
| MHQPHQPLPPTVM | 756.8776 | 1511.7377 | f(144-156) | / | / |
| MHQPHQPLPPTVMFPPQ | 661.3254 | 1982.3793 | f(144-160) | / | / |
| HQPHQPLPPT | 576.2953 | 1150.5883 | f(145-154) | / | / |
| HQPHQPLPPTVM | 691.3551 | 1370.6972 | f(145-156) | / | / |
| HQPHQPLPPTVMFPPQ | 617.6508 | 1849.9298 | f(145-160) | / | / |
| QPHQPLPPTVM | 622.8084 | 1243.6383 | f(146-156) | / | / |
| VMFPPQ | 359.6717 | 717.3520 | f(155-160) | / | / |

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|---|---|---|---|---|---|
| VMFPPQSVL | 1017.5629 | 1016.5365 | f(155-163) | / | / |
| FPPQSVL | 787.4301 | 786.4276 | f(157-163) | / | / |
| SQSKVLPVPQ | 541.8009 | 1071.6132 | f(166-175) | ACEi | Hayes et al., 2007 |
| QSKVLPVPQ | 995.5916 | 994.5811 | f(167-175) | / | / |
| QSKVLPVPQKAVPYPQR | 484.5272 | 1934.1102 | f(167-182) | / | / |
| SKVLPVPQ | 434.2542 | 866.5226 | f(168-175) | ACEi | Yamamoto et al., 1994 |
| KVLPVPQ | 390.7414 | 779.4905 | f(169-175) | ACEi | Maeno et al., 1996 |
| VLPVPQ | 652.3973 | 651.3956 | f(170-175) | / | / |
| KAVPYPQ | 401.7171 | 801.4385 | f(176-182) | / | / |
| KAVPYPQR | 479.7656 | 957.5396 | f(176-183) | / | / |
| RDMPIQA | 415.7139 | 829.4116 | f(183-189) | / | / |
| RDMPIQAF | 489.2411 | 976.4800 | f(183-190) | ACEi | Yamamoto et al., 1994 |
| RDMPIQAFLL | 602.3328 | 1202.6481 | f(183-192) | / | / |
| LYQEPVL | 861.4568 | 860.4644 | f(192-198) | / | / |
| LYQEPVLGPVRGPFP | 834.9509 | 1667.9035 | f(192-206) | / | / |
| LYQEPVLGPVRGPFPIIV | 997.5960 | 1993.1401 | f(192-209) | Immunomodulator | Boutrou et al., 2013 |
| YQEPVL | 748.3554 | 747.3803 | f(193-198) | / | / |
| YQEPVLGPVRGPFP | 778.4137 | 1554.8195 | f(193-206) | / | / |
| YQEPVLGPVRGPFPIIV | 941.0462 | 1880.0560 | f(193-209) | Immunomodulator | Boutrou et al., 2013 |
| QEPVL | 585.3251 | 584.3170 | f(194-198) | / | / |
| QEPVLGPVRGPFP | 696.8883 | 1391.7561 | f(194-206) | / | / |
| QEPVLGPVRGPFPII | 809.9563 | 1617.9243 | f(194-208) | / | / |
| QEPVLGPVRGPFPIIV | 859.4987 | 1716.9927 | f(194-209) | ACEi | Lu et al., 2016 |
| EPVLGPVRGPFP | 632.8584 | 1263.6976 | f(195-206) | ACEi | Hayes et al., 2007 |
| EPVLGPVRGPFPIIV | 795.4756 | 1588.9341 | f(195-209) | / | / |
| VLGPVRGPFPIIV | 682.4140 | 1362.8388 | f(197-209) | / | / |

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|---|---|---|---|---|---|
| LGPVRGPFPIIV | 632.8902 | 1263.7703 | f(198-209) | / | / |
| GPVRGPFP | 413.7223 | 825.4497 | f(199-206) | / | / |
| GPVRGPFPII | 526.8104 | 1051.6179 | f(199-208) | / | / |
| GPVRGPFPIIV | 576.3457 | 1150.6863 | f(199-209) | / | / |
| RGPFPIIV | 449.7709 | 897.5436 | f(202-209) | / | / |

[a]Abbreviations are: ACEi, angiotensin converting enzyme-inhibitory; DPPIV, dipeptidyl peptidase IV.
[b]One code letter was used for amino acid nomenclature.
[c]The observed mass is reported as $[M+nH]^{n+}$.
[d]The calculated mass is in Da.
[e]Potential bioactivities were retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

**Table S2**. αS1-casein-derived peptides identified in milk fermented with *Lactobacillus casei* PRA205 whole cells[a].

| *Sequence*[b] | *Observed mass (m/z)*[c] | *Calculated mass*[d] | *Fragment* | *Bioactivity*[e] | *Reference* |
|---|---|---|---|---|---|
| RPKHPIKH | 338.2150 | 1011.6090 | f(1-8) | / | / |
| RPKHPIKHQ | 570.8322 | 1139.6676 | f(1-9) | ACEi | Saito et al., 2000 |
| RPKHPIKHQGLPQ | 512.6430 | 1534.8844 | f(1-13) | / | / |
| RPKHPIKHQGLPQEVLN | 498.5285 | 1990.1224 | f(1-17) | / | / |
| KHPIKHQ | 296.4995 | 886.5137 | f(3-9) | / | / |
| GLPQEVLNE | 499.7432 | 997.5080 | f(10-18) | / | / |
| ENLLRF | 396.2126 | 790.4337 | f(18-24) | ACEi | Boutrou et al., 2013 |
| FVAPFPE | 806.3771 | 805.4010 | f(24-30) | / | / |
| FVAPFPEVF | 1052.5566 | 1051.5379 | f(24-32) | ACEi | Boutrou et al., 2013 |
| FVAPFPEVFGKE | 683.8712 | 1365.6969 | f(24-35) | / | / |
| VAPFPE | 659.3117 | 658.3326 | f(25-30) | / | / |
| VAPFPEVF | 453.2311 | 904.4695 | f(25-32) | ACEi | Boutrou et al., 2013 |
| VAPFPEVFGK | 545.7962 | 1089.5859 | f(25-34) | / | / |
| VAPFPEVFGKE | 610.2613 | 1218.6285 | f(25-35) | / | / |
| VFGKEKV | 403.7262 | 805.4698 | f(31-37) | / | / |
| VFGKEKVN | 307.4989 | 919.5127 | f(31-38) | / | / |
| VFGKEKVNEL | 581.8127 | 1161.6394 | f(31-40) | / | / |
| S(phospho)VEQKHIQ | 524.7428 | 1047.4750 | f(75-82) | / | / |
| RLKKYKVPQ | 387.2312 | 1158.7237 | f(100-108) | / | / |
| KKYKVPQ | 445.7761 | 889.5385 | f(102-108) | / | / |
| KYKVPQ | 381.7173 | 761.4436 | f(103-108) | / | / |
| LEIVPN | 684.3778 | 683.3854 | f(109-114) | / | / |
| S(phospho)AEELRH | 461.1781 | 920.3753 | f(115-121) | / | / |
| SMKEGIH | 401.1859 | 800.3851 | f(122-128) | / | / |

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|---|---|---|---|---|---|
| KEGIHAQ | 391.7040 | 781.4082 | f(124-130) | / | / |
| AQQKEPM | 416.1894 | 830.3956 | f(139-135) | / | / |
| QKEPMIGVN | 508.2554 | 1014.5168 | f(131-139) | / | / |
| FSDIPNPIGSE | 1175.5582 | 1174.5506 | f(179-189) | / | / |
| FSDIPNPIGSEN | 645.2999 | 1288.5935 | f(179-190) | / | / |
| FSDIPNPIGSENSE | 753.3396 | 1504.6682 | f(179-192) | / | / |
| FSDIPNPIGSENSEK | 817.3947 | 1632.7631 | f(179-193) | / | / |
| SDIPNPIGSENSE | 679.7936 | 1357.5997 | f(180-192) | / | / |
| DIPNPIGSENSE | 636.2773 | 1270.5677 | f(181-192) | / | / |

[a]Abbreviation is: ACEi, angiotensin converting enzyme-inhibitory.
[b]One code letter was used for amino acid nomenclature.
[c]The observed mass is reported as $[M+nH]^{n+}$.
[d]The calculated mass is in Da.
[e]Potential bioactivities were retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

**Table S3**. αS2-casein-derived peptides identified in milk fermented with *Lactobacillus casei* PRA205 whole cells.

| *Sequence*[a] | *Observed mass (m/z)*[b] | *Calculated mass*[c] | *Fragment* | *Bioactivity*[d] | *Reference* |
|---|---|---|---|---|---|
| SIIS(phospho)QETYK | 574.7641 | 1147.5162 | f(13-21) | / | / |
| RNAVPITPT | 484.7610 | 967.5451 | f(114-122) | / | / |
| NAVPITPT | 812.4495 | 811.4440 | f(115-122) | / | / |
| NAVPITPTLNRE | 662.8478 | 1323.7146 | f(115-126) | / | / |
| AVPITPT | 698.4023 | 697.4010 | f(116-122) | / | / |
| AVPITPTLNRE | 605.8537 | 1209.6717 | f(116-126) | / | / |
| LNREQLS(phospho)TS(phospho)EE | 733.2800 | 1464.5534 | f(123-133) | / | / |
| NSKKTVD | 396.2126 | 790.4185 | f(134-140) | / | / |
| MES(phospho)TEVFTK | 576.2371 | 1150.4617 | f(141-149) | / | / |
| TKKTKLTE | 474.7895 | 947.5651 | f(148-155) | / | / |
| TKVIPYVRYL | 417.9110 | 1250.7387 | f(198-207) | Antimicrobial | Alvarez-Ordóñez et al., 2013 |

[a]One code letter was used for amino acid nomenclature.
[b]The observed mass is reported as $[M+nH]^{n+}$.
[c]The calculated mass is in Da.
[d]Potential bioactivities were retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

**Table S4**. κ-casein-derived peptides identified in milk fermented with *Lactobacillus casei* PRA205 whole cells.

| *Sequence*[a] | *Observed mass (m/z)*[b] | *Calculated mass*[c] | *Fragment* | *Bioactivity*[d] | *Reference* |
|---|---|---|---|---|---|
| FSDKIA | 340.6720 | 679.3541 | f(18-23) | / | / |
| KYIPIQY | 462.7570 | 923.5116 | f(24-30) | / | / |
| KYIPIQYVL | 568.8331 | 1135.6641 | f(24-32) | / | / |
| SRYPSYGLN | 528.7613 | 1055.5036 | f(33-41) | / | / |
| YYQQKPV | 463.2334 | 924.4705 | f(42-48) | / | / |
| YYQQKPVAL | 555.2851 | 1108.5917 | f(42-50) | / | / |
| YYQQKPVALIN | 668.8545 | 1335.7187 | f(42-52) | / | / |
| YYQQKPVALINN | 725.8879 | 1449.7616 | f(42-53) | / | / |
| QKPVALINN | 498.7884 | 995.5764 | f(45-53) | / | / |
| NQFLPYPYYAKPA | 786.4022 | 1570.7820 | f(53-65) | / | / |
| QFLPYPYYAKPA | 729.3616 | 1456.7391 | f(54-65) | / | / |
| FLPYPYYAKPA | 665.3372 | 1328.7805 | f(55-65) | / | / |
| LPYPYYAKPA | 591.8096 | 1181.6121 | f(56-65) | / | / |
| YAKPA | 275.1487 | 548.2958 | f(61-65) | / | / |
| AVRSPA | 300.6684 | 599.3391 | f(66-71) | / | / |
| AVRSPAQIL | 477.7794 | 953.5658 | f(66-74) | / | / |
| AVRSPAQILQ | 541.8009 | 1081.6244 | f(66-75) | / | / |
| ARHPHPHLS | 351.1777 | 1050.5471 | f(96-104) | / | / |
| ARHPHPHLSF | 400.2009 | 1197.6156 | f(96-105) | / | / |
| ARHPHPHLSFM | 443.8951 | 1328.6560 | f(96-106) | / | / |
| DKTEIPTIN | 515.7586 | 1029.5342 | f(116-123) | / | / |
| KTEIPTIN | 458.2511 | 914.5073 | f(117-123) | / | / |
| EIPTIN | 686.3773 | 685.3646 | f(118-123) | / | / |
| TIASGEPT | 775.3848 | 774.3759 | f(124-131) | / | / |

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|---|---|---|---|---|---|
| VATLEDS(phospho)PE | 520.7034 | 1039.4111 | f(143-152) | / | / |
| VIESPPEIN | 997.5065 | 996.5128 | f(152-160) | / | / |
| SPPEIN | 656.3215 | 655.3177 | f(155-160) | / | / |
| SPPEINTVQ | 984.5185 | 983.4924 | f(155-163) | / | / |
| VTSTAV | 577.3151 | 576.3119 | f(164-169) | / | / |

[a]One code letter was used for amino acid nomenclature.
[b]The observed mass is reported as $[M+nH]^{n+}$.
[c]The calculated mass is in Da.
[d]Potential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

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**Table S5**. β-casein-derived peptides identified in milk fermented with *Lactobacillus rhamnosus* PRA331 whole cells[a].

| *Sequence*[b] | *Observed mass (m/z)*[c] | *Calculated mass*[d] | *Fragment* | *Bioactivity*[e] | *Reference* |
|---|---|---|---|---|---|
| LNVPGEIVE | 485.2516 | 968.5179 | f(6-14) | ACEi | Gobbetti et al., 2000 |
| NVPGEIVE | 856.4256 | 855.4338 | f(7-14) | / | / |
| SITRIN | 352.1995 | 702.4024 | f(22-27) | / | / |
| KKIEKF | 396.7440 | 791.4905 | f(28-33) | / | / |
| KKIEKFQS(phospho)E | 608.8120 | 1215.5900 | f(28-36) | / | / |
| DKIHPF | 378.6905 | 755.3966 | f(47-52) | ACEi | Gobbetti et al., 2000 |
| DKIHPFA | 414.2081 | 826.4337 | f(47-53) | / | / |
| SLVYPFPGPIPN | 1300.6991 | 1299.6863 | f(57-68) | / | / |
| LVYPFPGPIPN | 1213.6643 | 1212.6543 | f(58-68) | / | / |
| VYPFPGPIPN | 1100.5503 | 1099.5702 | f(59-68) | ACEi Antioxidant, Opioid | Eisele et al., 2013 |
| VYPFPGPIPNSLPQ | 763.3809 | 1524.7977 | f(59-72) | / | / |
| YPFPGPIPN | 1001.5294 | 1000.5018 | f(60-68) | ACEi DPPIV-inhibitor, Opioid | Saito et al., 2000 |
| SLPQNIPPL | 978.5704 | 977.5546 | f(69-77) | / | / |
| SLPQNIPPLTQTPV | 752.9436 | 1503.8297 | f(69-82) | / | / |
| SLPQNIPPLTQTPVVVPPFLQPEVM | 1371.2513 | 2740.4874 | f(69-93) | / | / |
| QNIPPLTQTPV | 604.3167 | 1206.6608 | f(72-82) | / | / |
| QNIPPLTQTPVVVPPF | 874.0031 | 1745.9716 | f(72-87) | / | / |
| QNIPPLTQTPVVVPPFLQPE | 738.7323 | 2213.2096 | f(72-91) | / | / |
| QNIPPLTQTPVVVPPFLQPEVM | 815.4550 | 2443.3185 | f(72-93) | / | / |
| QNIPPLTQTPVVVPPFLQPEVMGVS | 896.4847 | 2686.4404 | f(69-96) | / | / |
| NIPPLTQTPV | 540.3031 | 1078.6023 | f(73-82) | ACEi | Gobbetti et al., 2000 |

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|---|---|---|---|---|---|
| NIPPLTQTPVVVPPF | 809.9655 | 1617.9131 | f(73-87) | / | / |
| NIPPLTQTPVVVPPFLQPEVM | 1158.6488 | 2315.2599 | f(73-93) | / | / |
| TQTPVVVPPF | 542.7930 | 1083.5965 | f(78-87) | / | / |
| TQTPVVVPPFL | 599.3400 | 1196.6805 | f(78-88) | / | / |
| TQTPVVVPPFLQPE | 776.4225 | 1550.8345 | f(78-91) | / | / |
| TQTPVVVPPFLQPEVM | 891.4927 | 1780.9434 | f(78-93) | / | / |
| TQTPVVVPPFLQPEVMGVS | 1013.0422 | 2024.0653 | f(78-96) | / | / |
| TQTPVVVPPFLQPEVMGVSKVKEAMAP | 960.5032 | 2878.5337 | f(78-104) | / | / |
| QTPVVVPPFLQPE | 725.8957 | 1449.7868 | f(79-91) | / | / |
| QTPVVVPPFLQPEVM | 840.9478 | 1679.8957 | f(79-93) | / | / |
| PVVVPPFLQPE | 611.3391 | 1220.6805 | f(81-91) | / | / |
| PVVVPPFLQPEVM | 726.3960 | 1450.7894 | f(81-93) | / | / |
| VVPPFLQPE | 1025.5809 | 1024.5593 | f(83-91) | / | / |
| VVPPFLQPEVM | 1255.6756 | 1254.6682 | f(83-93) | / | / |
| VPPFLQPEVM | 578.7956 | 1155.5998 | f(84-93) | / | / |
| PEVMGVSKVKEAMAPK | 567.6384 | 1700.9074 | f(90-105) | / | / |
| VMGSKVKEA | 349.8603 | 1046.5794 | f(92-101) | / | / |
| MAPKHKEMPFPKYPVEPF | 725.0518 | 2172.0900 | f(102-119) | / | / |
| APKHKEMPFPKYPVEPF | 681.3265 | 2041.0495 | f(103-119) | / | / |
| HKEMPFPKYPVEPF | 582.6204 | 1744.8647 | f(106-119) | / | / |
| EMPFPKYPVEPF | 740.8436 | 1479.7108 | f(108-119) | / | / |
| MPFPKYPVEP | 602.8002 | 1203.5998 | f(109-118) | / | / |
| MPFPKYPVEPF | 676.3260 | 1350.6682 | f(109-119) | / | / |
| MPFPKYPVEPFTE | 791.3602 | 1580.7585 | f(109-121) | / | / |
| NLHLPLP | 402.2289 | 802.4701 | f(132-138) | ACEi | Kohmura et al., 1989 |
| NLHLPLPL | 458.7739 | 915.5542 | f(132-139) | / | / |

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|---|---|---|---|---|---|
| NLHLPLPLL | 515.3360 | 1028.6632 | f(132-140) | ACEi | Robert et al., 2004 |
| NLHLPLPLLQ | 579.3461 | 1156.6968 | f(132-141) | / | / |
| NLHLPLPLLQS | 622.8557 | 1243.7288 | f(132-142) | / | / |
| NLHLPLPLLQSW | 715.8693 | 1429.8082 | f(132-143) | / | / |
| LHLPLP | 345.2061 | 688.4272 | f(133-138) | ACEi | Kohmura et al., 1989 |
| LHLPLPL | 401.7568 | 801.5112 | f(133-139) | ACEi | Quiros et al., 2007 |
| LHLPLPLLQ | 522.3205 | 1042.6539 | f(133-141) | / | / |
| LHLPLPLLQS | 565.8474 | 1129.6859 | f(133-142) | / | / |
| LHLPLPLLQSW | 658.8784 | 1315.7652 | f(133-143) | / | / |
| HLPLPL | 345.2061 | 688.4272 | f(134-139) | / | / |
| HLPLPLLQSW | 602.3478 | 1202.6812 | f(134-143) | / | / |
| LPLPLLQ | 793.5105 | 792.5109 | f(135-141) | / | / |
| LPLPLLQSW | 533.8107 | 1065.6223 | f(135-143) | / | / |
| WMHQPHQPLPPTVMFPPQ | 723.3596 | 2167.0496 | f(143-160) | / | / |
| MHQPHQPLPPT | 641.8081 | 1281.6288 | f(144-154) | / | / |
| MHQPHQPLPPTVM | 504.9035 | 1511.7377 | f(144-156) | / | / |
| MHQPHQPLPPTVMFPPQ | 661.3167 | 1980.9703 | f(144-160) | / | / |
| HQPHQPLPPT | 576.2926 | 1150.5883 | f(145-154) | / | / |
| HQPHQPLPPTVM | 461.2329 | 1380.6972 | f(145-156) | / | / |
| HQPHQPLPPTVMFPPQ | 617.6437 | 1849.9298 | f(145-160) | / | / |
| FPPQSVL | 787.4370 | 786.4272 | f(157-163) | / | / |
| SQSKVLPVPQ | 541.8019 | 1081.6132 | f(166-175) | ACEi | Hayes et al., 2007 |
| QSKVLPVPQ | 498.2873 | 994.5811 | f(167-175) | / | / |
| SKVLPVPQ | 434.2558 | 866.5226 | f(168-175) | ACEi | Yamamoto et al., 1994 |
| KVLPVPQ | 780.4975 | 779.4905 | f(169-175) | ACEi | Maeno et al., 1996 |
| VLPVPQ | 652.3929 | 651.3956 | f(170-175) | / | / |

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|---|---|---|---|---|---|
| KAVPYPQ | 401.7177 | 801.4385 | f(176-182) | / | / |
| KAVPYPQRDMPI | 707.8531 | 1413.7438 | f(176-186) | / | / |
| RDMPIQAF | 489.2344 | 976.4800 | f(183-190) | ACEi | Yamamoto et al., 1994 |
| RDMPIQAFL | 545.7702 | 1089.5641 | f(183-191) | / | / |
| RDMPIQAFLL | 602.3312 | 1202.6481 | f(183-192) | / | / |
| LYQEPVL | 861.4602 | 860.4644 | f(192-198) | / | / |
| YQEPVL | 748.3849 | 747.3803 | f(193-198) | / | / |
| YQEPVLGPVRGPFP | 778.4100 | 1554.8195 | f(193-206) | / | / |
| YQEPVLGPVRGPFPIIV | 941.0424 | 1880.0560 | f(193-209) | Immunomodulator | Boutrou et al., 2013 |
| QEPVLGPVRGPFP | 686.8812 | 1391.7561 | f(194-206) | / | / |
| QEPVLGPVRGPFPIIV | 859.5135 | 1716.9927 | f(194-209) | ACEi | Lu et al., 2016 |
| EPVLGPVRGPFP | 632.8351 | 1263.6976 | f(195-206) | ACEi | Hayes et al., 2007 |
| EPVLGPVRGPFPIIV | 795.4796 | 1588.9341 | f(195-209) | / | / |
| VLGPVRGPFPIIV | 682.4057 | 1362.8388 | f(197-209) | / | / |
| LGPVRGPFPIIV | 632.8861 | 1263.7703 | f(198-209) | / | / |
| GPVRGPFP | 413.7160 | 825.4497 | f(199-206) | / | / |
| GPVRGPFPII | 526.7979 | 1051.6179 | f(199-208) | / | / |
| GPVRGPFPIIV | 576.3487 | 1150.6863 | f(199-209) | / | / |
| RGPFPIIV | 449.7698 | 897.5436 | f(202-209) | / | / |

[a]Abbreviations are: ACEi, angiotensin converting enzyme-inhibitory; DPPIV, dipeptidyl peptidase IV.
[b]One code letter was used for amino acid nomenclature.
[c]The observed mass is reported as $[M+nH]^{n+}$.
[d]The calculated mass is in Da.
[e]Potential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

**Table S6**. αS1-casein-derived peptides identified in milk fermented with *Lactobacillus rhamnosus* PRA331 whole cells[a].

| *Sequence*[b] | *Observed mass (m/z)*[c] | *Calculated mass*[d] | *Fragment* | *Bioactivity*[e] | *Reference* |
|---|---|---|---|---|---|
| RPKHPIKH | 338.1924 | 1011.6090 | f(1-8) | / | / |
| RPKHPIKHQ | 380.8905 | 1139.6676 | f(1-9) | ACEi | Saito et al., 2000 |
| RPKHPIKHQGLPQ | 512.6139 | 1534.8844 | f(1-13) | / | / |
| RPKHPIKHQGLPQEVLN | 498.5303 | 1990.1224 | f(1-17) | / | / |
| KHPIKHQ | 444.2507 | 886.5137 | f(3-9) | / | / |
| GLPQEVL | 755.4085 | 754.4298 | f(10-16) | / | / |
| GLPQEVLNE | 499.7582 | 997.5080 | f(10-18) | / | / |
| ENLLRF | 396.2095 | 790.4337 | f(18-24) | ACEi | Boutrou et al., 2013 |
| FVAPFPE | 806.4074 | 805.4010 | f(24-30) | / | / |
| FVAPFPEVF | 1052.5164 | 1051.5379 | f(24-32) | ACEi | Boutrou et al., 2013 |
| FVAPFPEVFGKE | 683.8608 | 1365.6969 | f(24-35) | / | / |
| VAPFPE | 659.3349 | 658.3326 | f(25-30) | / | / |
| VAPFPEVF | 905.4909 | 904.4695 | f(25-32) | ACEi | Boutrou et al., 2013 |
| VAPFPEVFGK | 545.8130 | 1089.5859 | f(25-34) | / | / |
| VAPFPEVFGKE | 610.3173 | 1218.6285 | f(25-35) | / | / |
| APFPEVF | 806.4074 | 805.4010 | f(26-32) | / | / |
| APFPEVFGKE | 560.7993 | 1119.5601 | f(26-35) | / | / |
| VFGKEKVN | 460.7541 | 919.5127 | f(31-38) | / | / |
| KKYKVPQ | 445.7685 | 889.5385 | f(102-108) | / | / |
| KYKVPQ | 381.7245 | 761.4436 | f(103-108) | / | / |
| LEIVPN | 684.3640 | 683.3854 | f(109-114) | / | / |
| S(phospho)AEELRH | 461.1787 | 920.3753 | f(115-121) | / | / |
| S(phospho)AEELRHSM | 570.2206 | 1138.4478 | f(115-123) | / | / |
| KEGIHAQ | 391.6973 | 781.4082 | f(124-130) | / | / |

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|---|---|---|---|---|---|
| APSFSDIPNPIGSENSE | 880.9123 | 1759.7901 | f(176-192) | / | / |
| FSDIPNPIGSE | 588.2765 | 1174.5506 | f(179-189) | / | / |
| FSDIPNPIGSEN | 645.3093 | 1288.5935 | f(179-190) | / | / |
| FSDIPNPIGSENSE | 753.3208 | 1504.6682 | f(179-192) | / | / |
| IPNPIGSENSE | 578.7531 | 1155.5408 | f(182-192) | / | / |

[a]Abbreviation is: ACEi, angiotensin converting enzyme-inhibitory.
[b]One code letter was used for amino acid nomenclature.
[c]The observed mass is reported as $[M+nH]^{n+}$.
[d]The calculated mass is in Da.
[e]Potential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

**Table S7**. αS2-casein-derived peptides identified in milk fermented with *Lactobacillus rhamnosus* PRA331.

| *Sequence*[a] | *Observed mass (m/z)*[b] | *Calculated mass*[c] | *Fragment* | *Bioactivity*[d] | *Reference* |
|---|---|---|---|---|---|
| SIIS(phospho)QETYK | 574.7617 | 1147.5162 | f(13-21) | / | / |
| NAVPITPT | 812.4463 | 811.4440 | f(115-122) | / | / |
| NAVPITPTLN | 520.2697 | 1038.5710 | f(115-124) | / | / |
| NAVPITPTLNRE | 662.8581 | 1323.7146 | f(115-126) | / | / |
| AVPITPT | 698.4069 | 697.4010 | f(116-122) | / | / |
| AVPITPTLNRE | 605.8419 | 1209.6717 | f(116-126) | / | / |
| MES(phospho)TEVFTK | 576.2277 | 1150.4617 | f(141-149) | / | / |
| MES(phospho)TEVFTKK | 640.2751 | 1278.5567 | f(141-150) | / | / |
| TKVIPYVRYL | 417.9150 | 1250.7387 | f(198-207) | Antimicrobial | Alvarez-Ordóñez et al., 2013 |

[a]One code letter was used for amino acid nomenclature.
[b]The observed mass is reported as $[M+nH]^{n+}$
[c]The calculated mass is in Da.
[d]Potential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

**Table S8**. κ-casein-derived peptides identified in milk fermented with *Lactobacillus rhamnosus* PRA331 whole cells.

| *Sequence*[a] | *Observed mass (m/z)*[b] | *Calculated mass*[c] | *Fragment* | *Bioactivity*[d] | *Reference* |
|---|---|---|---|---|---|
| FSDKIA | 340.6721 | 679.3541 | f(18-23) | / | / |
| KYIPIQY | 462.7544 | 923.5116 | f(24-30) | / | / |
| KYIPIQYVL | 568.8299 | 1135.6641 | f(24-32) | / | / |
| KYIPIQYVLS | 612.3488 | 1222.6961 | f(24-33) | / | / |
| SRYPSYGLN | 528.7591 | 1055.5036 | f(33-41) | / | / |
| RYPSYGLN | 485.2353 | 968.4716 | f(34-41) | / | / |
| YYQQKPVAL | 555.2734 | 1108.5917 | f(42-50) | / | / |
| YYQQKPVALIN | 668.8605 | 1335.7187 | f(42-52) | / | / |
| YYQQKPVALINN | 725.8975 | 1449.7616 | f(42-53) | / | / |
| QQKPVALINN | 562.8214 | 1123.6349 | f(44-53) | / | / |
| QKPVALINN | 498.7893 | 995.5764 | f(45-53) | / | / |
| QFLPYPYYAKPA | 729.3794 | 1456.7391 | f(54-65) | / | / |
| FLPYPYYAKPA | 665.3492 | 1328.6805 | f(55-65) | / | / |
| LPYPYYAKPA | 591.8004 | 1181.6121 | f(56-65) | / | / |
| AVRSPA | 300.6625 | 599.3391 | f(66-71) | / | / |
| AVRSPAQIL | 477.7849 | 953.5658 | f(66-74) | / | / |
| AVRSPAQILQ | 541.8019 | 1081.6244 | f(66-75) | / | / |
| ARHPHPHLS | 351.1755 | 1050.5471 | f(96-104) | / | / |
| ARHPHPHLSFM | 443.8816 | 1328.6560 | f(96-106) | / | / |
| EIPTIN | 686.3505 | 685.3646 | f(118-123) | / | / |
| TIASGEPT | 775.3862 | 774.3759 | f(124-131) | / | / |
| VATLEDS(phospho)PE | 520.7108 | 1039.4111 | f(143-152) | / | / |
| VIESPPEIN | 499.2569 | 996.5128 | f(152-160) | / | / |
| SPPEIN | 328.6530 | 655.3167 | f(155-160) | / | / |

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|---|---|---|---|---|---|
| SPPEINTVQ | 492.7466 | 983.4924 | f(155-163) | / | / |

[a]One code letter was used for amino acid nomenclature.
[b]The observed mass is reported as $[M+nH]^{n+}$.
[c]The calculated mass is in Da.
[d]Potential bioactivities were achieved retrieved by searching peptide sequences against the BIOPEP and MBPDB databases (Minkiewicz et al., 2008; Nielsen et al., 2017).

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