Bioaccessible peptides released by in vitro gastrointestinal digestion of fermented goat milks

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Keywords:	Fermented goat´s milk, Bioaccessible peptides, Tandem mass spectrometry, Gastrointestinal digestion, Peptidomics

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Referee comments and answers

Referee A:

The authors used a peptidomic method to identify the peptides products by St and St+LP. during the manufacturing of fermented goat milk. The authors also evaluate the resistance and bioaccessible peptides by using in vitro gastrointestinal digestion and LC-MS/MS.
I have a few comments:
1,The identified peptides from in vitro gastrointestinal digestion are significantly less than those of in the goat milk fermented with the classical St and St+LP (151 VS 232). What is the reason for this? In general, in vitro gastrointestinal digestion should produce more peptides than fermentation of milk.
Please discuss.
The high number of different peptides recovered in the non–digested fermented products resulted from the proteolytic action of the fermenting strains, with very diverse cleavage sites. We agree with the idea that digestion will produce more peptides. Hence, although the number of peptides in the *in vitro* digests was increased, since the cleavages exclusively arise from the pepsin, trypsin and chymotrypsin action, their diversity was reduced. In that way the number of different sequences has been decreased in the digests. We have previously observed this behavior in the digestion of blue cheese, which shows a high degree of proteolysis. The comparison of the peptidome before and after digestion showed a higher homology between sequences after digestion, and resulted in a lower number of different sequences than expected (Sánchez-Rivera et al. Electrophoresis 2014, 35, 1627-1636). In this regard, other authors have reported a lower number of experimentally observed peptides in relation to the those expected upon *in vitro* digestion (Tonda et al. Food & Function, 2017). This consideration has been added in page 14.

2, The authors stated in the manuscript (page 10, paragraph1) that for beta-casein, the highest abundance of peptides corresponded to the 180-207 region, however from the table, there is no abundance information given. Are the abundance calculated from numbers of peptide identified or MS/MS spectrum?
Yes, the abundance of peptides corresponds to the number of peptides released from a particular part of the protein, and this was very marked in the case of the 180-207 region. In order to improve clarity, the term has been replaced by "number of peptides". In addition, a table with the peptides released from beta-casein has been included in the Electronic supplementary material.

3, for αs1-casein, the absence of peptide from regions (41-99) and (152-159) could be resistant to hydrolysis, and it also could be due to heavily phosphorylation and not retained by cation ion exchange and/or not detected by mass spectrometer due to low ionization efficiency. Please discuss.
The lower number of peptides from the cited regions in α_{s1}-casein might derive from the phosphorylated serines in the case of the 41-99 region. It is known that in a complex mixture, phosphorylated peptides are comparatively less ionized. Conversely, no phosphorylation site is found in the 152-159 region. Miclo and others (2012) suggested the hydrophobicity of the different protein regions but concluded that accessibility to substrate appeared as the crucial parameter to determine casein susceptibility to hydrolysis by the proteolytic system of *S. thermophilus*. Therefore, the contribution or more than one factor is feasible. The discussion in page 11 has been modified accordingly.

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Referee B:

The manuscript is an interesting application of comparative proteomics (or specifically, peptidomics) in a food and nutritional application. Overall, I thought the manuscript was well-written and the discussions were applicable to the results and very much to the point. However, there are several instances where the writing has awkward English phrasing but it is still understandable.
In order to improve the manuscript, a language revision by an English native speaker has been conducted.

A few technical issues to address:

1) The authors refer to "identified" peptides frequently. However, as with any proteomic investigation, identifications are never absolute without a significant level of validation (such as by using peptide standards, etc). Therefore, the authors should introduce the necessary uncertainty in their discussion by potentially listing identification scores when applicable and changing their terminology to "potential identifications" or similar language.

We agree in the need to show the level of confidence in the peptide identification. A sentence with this purpose has been added in the Materials and Methods section (Analysis by LC-MS/MS). Besides, in order to take into account the uncertainty in the identification, the term potential identification has been introduced in some sentences along the manuscript, as suggested by the reviewer.

2) The authors are using a BCA assay calibrated with BSA to quantify and compare the "protein concentration" in enzymatically-digested samples. The samples are a highly heterogenous mixture of small to large peptides, partially digested proteins, and minimally- to non-digested proteins. The BCA signal response for such highly heterogeneous mixtures will not accurately represent the total concentration of all the components present because the assay response will be different for different component of the mixture. Comparison of the BCA assay results from two different heterogeneous mixtures will not provide a meaningful results if two two mixtures are not similar in composition. For the samples analyzed in this work, their mixtures are not similar in composition as reported in the manuscript. Therefore, comparisons of BCA-derived protein concentration, as found in the "peptide profile analysis" section and in Table 3 are meaningless. The discussion of protein content should be removed from the manuscript.

The BCA assay was intended to have a rough idea about the protein composition, being aware of the samples heterogeneity. We agree with the reviewer in the non-accurate approach to measure the protein content in the different digestion fractions. Therefore, we have eliminated the table and sentence on protein content.

3) It is also my belief that Table 1 could be moved to an electronic supplementary material file.

As suggested by the reviewer, Table 1 has been moved to an electronic supplementary material file. Moreover, a table with peptides released from beta-casein has been added.

Abstract

In this study, ultrafiltered goat milks fermented with the classical starter bacteria *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarus* subsp. *thermophilus* or with the classical starter plus the *Lactobacillus plantarum* C4 probiotic strain were analyzed using ultra-high performance liquid chromatography-quadrupole-time-of-flight tandem mass spectrometry (UPLC-Q-TOF-MS/MS) and/or high performance liquid chromatography-ion trap (HPLC-IT-MS/MS). Partial overlapping of the identified sequences with regard to fermentation culture was observed. Evaluation of the cleavage specificity suggested a lower proteolytic activity of the probiotic strain. Some of the potentially identified peptides had been previously reported as angiotensin converting enzyme (ACE)-inhibitory, antioxidant and antibacterial and might account for the *in vitro* activity previously reported for these fermented milks. Simulated digestion of the products was conducted in presence of a dialysis membrane to retrieve the bioaccessible peptide fraction. Some sequences with reported physiological activity resisted digestion but were found in the non-dialyzable fraction. However, new forms released by digestion, such as the antioxidant α_{s1}-casein ^{144}YFYPQL^{149}, the antihypertensive α_{s2}-casein ^{90}YQKFPQY^{96} and the antibacterial α_{s2}-casein ^{165}LKKISQ^{170} were found in the dialyzable fraction of both fermented milks. Moreover, in the fermented milk including the probiotic strain, the k-casein dipeptidyl peptidase IV inhibitor (DPP-IV) ^{51}INNQFLPYPY^{60} as well as additional ACE-inhibitory or antioxidant sequences could be identified. With the aim to anticipate further biological outcomes, quantitative structure activity relationship (QSAR) analysis was applied to the bioaccessible fragments and led to propose potential ACE inhibitory sequences.

Keywords: Fermented goat´s milk; Bioaccessible peptides; Tandem mass spectrometry; Gastrointestinal digestion; Peptidomics

Introduction

Fermented milk products have a long history of being beneficial to human health. The physiological effects are often attributed to the action of probiotic microflora in the product. In milk fermentation the involved metabolites contribute to confer chemical, biochemical and nutritional attributes [1]. The proteolytic system of lactic-acid bacteria comprises extracellular cell-wall bound proteinases that initiate the degradation of milk proteins into oligopeptides, peptide transporters that take up the peptides into the cell, and various intracellular peptidases that degrade the peptides into shorter peptides and amino acids [2]. This can lead to the release of peptides with bioactive properties from fermented dairy products [3]. The proteolytic activity is influenced by the type of dairy product, the technology adopted and, specially, the bacterial strain [4]. In some cases, a combination of selected yeasts and lactic acid bacteria is used with the aim to generate peptides with known health benefits. Thus, a screening with lactic acid bacteria lead to select a mixed starter containing *Streptococcus thermophilus* and different *Lactobacillus* strains (casei, helveticus, plantarum) to produce goats' milk with γ-aminobutyric acid (GABA) and angiotensin-I converting enzyme (ACE)-inhibitory peptides [5]. Caprine milk fermentation products, such as kefir, have been the source of ACE inhibitory peptides [6, 7]. We have recently demonstrated that fermentation of ultrafiltered goat's milk with lactic acid bacteria including *Lactobacillus plantarum* C4, a strain with demonstrated probiotic activity in terms of *in vitro* intestinal microbiota modulation [8], results in the development several biological activities [9]. In this regard, the proteolytic nature and ability to generate bioactive peptides of this strain remained to be investigated.

There is a general agreement that caprine milk is more easily digested than bovine milk, a major factor affecting digestibility being the size of the lipid globules

[10]. Bovine and caprine milk differ in protein composition and the lower content of α_{s1}-casein in goat's milk has been associated with its lower allergenicity. Moreover, the content in this protein is related to milk coagulation properties, which influence protein digestibility. Studies on the peptides released after the simulated gastrointestinal digestion of goat milk proteins have shown the release of ACE [11], [12] and dipeptidyl peptidase IV (DPP-IV) inhibitory sequences [13] as well as antibacterial [14] and antioxidant peptides [15]. Interestingly, in fermented goat's milk products, such as cheese, some peptides with reported physiological activities have shown resistance to *in vitro* gastrointestinal digestion, such as the antihypertensive peptide β-casein, ^{133}LHLPLP^{138} [16].

The objective of this research was to identify the peptides produced by *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* with or without co-culture with the probiotic strain *Lactobacillus plantarum* C4 during the manufacturing of fermented goat milks. *In vitro* gastrointestinal digestion in combination with dialysis was conducted to evaluate the resistance and bioaccesibility of the released protein fragments. Since the assayed fermented milks had previously shown antioxidant, ACE-inhibitory and antimicrobial activity, comparison of the resistant sequences with those reported in the literature in combination of computer-assisted prediction for ACE inhibition was applied to identify most possible bioactive peptide sequences in the fermented goat milks.

Materials and methods

Chemicals and samples

Raw goat milk samples from Murciano-Granadina breed were collected from a farm in the region of Granada (Spain). They were skimmed by centrifugation and concentrated

by ultrafiltration through a 50 kDa membrane (Vivaflow 2000, Sartorius Stedin Biotech, Madrid, Spain). Fermentation was conducted with (i) the classical starter bacteria *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus salivarius* subsp. *thermophile* (St) and (ii) St + the probiotic strain *Lactobacillus plantarum* C4 [8] (St+ LP). Enzymes and bile salts were purchased from Sigma Chemical Co (St Louis, MO, USA), porcine pepsin (P-7000), porcine pancreatin (P-1500) and porcine bile extract (B-8631). All other reagents such as HCl, ammonia, $NaHCO_3$, formic acid, acetonitrile, were purchased from Sigma Chemical.

Isolation of peptide fractions

Fermented goat milk samples were isolated based on the method developed by [17] with some modifications. In the first step, samples were centrifuged at 3,000 *g* for 30 min at 4 ºC (Sigma 2-16PK, Sartorius, Goettingen, Germany). The precipitate was discarded and the supernatant was adjusted to pH 2.0 by addition of HCl. In the second step, the acidified supernatant was filtered through a 30 kDa cut off ultrafiltration membrane (Vivaspin20, Sartorius) and 100 ml of the filtrate was applied to a Dowex 50 WX2 cation exchange column (2.6 x 10 cm, H+-form, 200-400 mesh, Serva, Heidelberg, Germany). After washing with 60 ml of Milli-Q water, peptides were eluted with 200 ml of 2M aqueous ammonia. Ammonia was firstly evaporated *in vacuo* and then samples were freeze dried. This procedure was carried out by duplicate.

***In vitro* gastrointestinal digestion of the fermented goat milk samples**

Fermented goat milk samples were subjected to *in vitro* gastrointestinal digestion in duplicate as described by [18]. Briefly, 20 g of each fermented milk were homogenized with 60 ml of Milli-Q water and subjected to gastric digestion with pepsin and duodenal

digestion with pancreatin/bile solution. To stop intestinal digestion, samples were immersed in a water-bath at 100 ºC for 5 min. The digests were centrifuged at 3,500 *g* for 1 hour at 4 ºC and the supernatants, the soluble fraction, were freeze dried and kept until the analysis. The dialysis assay was carried out according to [18] to identify the potential bioaccessible peptides. It comprised a gastric step followed by an intestinal step where dialysis was included (dialysis bag: molecular weight 12-14 kDa; Visking 45 mm x 27 mm, Medicell International, London, UK). Dialysis tubing, containing 25 ml of bidistilled deionized water and an amount of $NaHCO_3$ equivalent to titratable acidity measured previously, were placed in the flasks together with 20 g aliquots of the pepsin digest and incubated in the shaken bath at 37 ºC for 30 min. An amount of freshly prepared pancreatin-bile extract mixture (0.001 g pancreatin and 0.006 g bile sals/samples) was added to the flask and the incubation continued up to 2 h. Dialyzable and non-dialyzable fractions were weighted, freeze dried and stored until the assay.

Total soluble protein content

The total protein content of the samples was determined based on the bicinchoninic acid assay according to the instructions of Thermo Scientific™ Pierce™ BCA™ Protein Assay kit, in a 96 well plate using a FLUOStar Omega microplate reader (BMG Labtech, Germany). Serial dilutions with bovine serum albumin (provided with the kit) were used as standard. Results were expressed as mg/mL.

Analysis by on-line reverse-phase high performance liquid chromatography tandem mass spectrometry (LC-MS/MS)

Before injection, fermented goat milk samples after ion exchange and digested samples were dissolved in water with formic acid (0.1%) at 2 mg/mL protein concentration and

centrifuged at 10,000*g* to precipitate all impurities. If turbidity was shown, also a filtration step through 0.45 µm µm size pore filters (Millex®-GS, Merck Millipore Ltd., Cork, Ireland) was carried out.

Chromatographic analysis of the samples was performed with an Acquity UPLC® system (Waters Technologies, Cerdanyola del Vallès, Spain) with an Acquity UPLC BEH 130 column, a C18 column 100 mm of length, 2.1 mm of internal diameter, 1.7 µm of particle size and 130 Å of pore diameter (Waters Technologies, Cerdanyola del Vallès, Spain). The UPLC system was connected online to a quadrupole-time of flight MS/MS detector, equipped with an electrospray ionization source (Bruker Daltonik, Bremen, Germany). Solvent A was water with 0.1 % formic acid and solvent B was acetonitrile with 0.1% formic acid and the flow used was 0.2 mL/min. The peptide fractions were eluted with an isocratic gradient after 1 min of pure solvent A, up to 35 % B within 28 min, then in 2 min 70 % of solvent B was reached and maintained during 2.5 minutes. The injection volume was 15 µL and the absorbance was monitored at 214 nm. The nebulizer pressure was set at 2 bar, the temperature of the source at 180 ºC and the capillary voltage at 4.5 kV Spectra were recorded over the mass/charge (m/z) range 50-1500 and 3 spectra were averaged in the MS analyses. The signal threshold to perform auto MS (n) analyses was 5,000 counts and three precursor ions were isolated within a range of 100-1500 m/z and fragmented with a voltage ramp depending of the isolation mass of the precursor ion, from 20 to 70eV.

Alternatively, the analyses were performed on an Agilent 1100 HPLC system (Agilent Technologies, Waldbronn, Germany) followed by on-line MS/MS analysis on an ion trap instrument (Esquire 3000, Bruker Daltonik GmbH, Bremen, Germany) as previously described [16]. Chromatographic separations were performed with a Mediterranea Sea18 150 mm × 2.1 mm column (Teknokroma, Barcelona, Spain).

Samples were injected at a protein concentration of 1 mg/mL, the flow rate was 0.2 mL/min and the injection volume was 50 µL. Peptides were eluted with a linear gradient from 10 % to 55 % of solvent B (acetonitrile: formic acid 0.1 %) and 45 % solvent A (water: formic acid 0.1%) in 95 min. Data Analysis (version 4.0; Bruker Daltoniks) was used to process and transform spectra. Data were processed with Data Analysis TM (version 4.0, Bruker Daltonik, Bremen, Germany). The m/z spectral data were processed with Biotools (Version 3.2, Bruker Daltonik, Bremen, Germany) where the deconvoluted mass spectra were matched against a homemade database with the main goat milk proteins (α_{s1}-casein, α_{s2}-casein, β-casein, κ-casein, α-lactoalbumin and β-lactoglobulin) sequences retrieved from the UniprotKB database (uniprot.org). Peptide sequencing was performed by MASCOT (matrixscience.com) with error tolerances 0.1 % for precursor masses and 0.5 Da for fragment masses. Only individual scores indicating identity or extensive homology (P<0.05) were used. Besides, the matched MS/MS spectra were interpreted by using BioTools version 3.2 (Bruker).

Peptide profile analysis

Venn's diagrams were executed with Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html). The Enzyme Predictor tool [19] was used to analyze protein cleavages in the peptides. The identified peptides were searched against the BIOPEP bioactive peptide database [20]. The AHT pin *in silico* platform (http://crdd.osdd.net/raghava/ahtpin/#) [21] was used in the variable length mode, amino acid composition model, and the SVM threshold was set to 0.9.

Results and discussion

Peptide profile analysis

The fermented milks were submitted to cation exchange at pH 2 followed by elution with ammonia in order to recover the whole peptide fraction. Fermented milk with St showed a higher protein concentration (0. 013 ± 0.003) than St+LP (0.009 ± 0.002). These values might account for the fermentation differences with more protein coagulation and less soluble protein/peptides in the case of St+LP, as previously observed [9]. A combination of two complementary LC-MS/MS settings to cover a wide range of sequence lengths permitted the potential identification of a total of 232 different peptides in St and St+LP fermented goat milks. From these, 46 % corresponded to β-casein, 24 % to α_{s2}-casein, 18 % to α_{s1}-casein and 13 % to k-casein. This distribution was compared with the content of the different caseins in goat's milk. In contrast to cow's milk, where the most abundant protein is α_{s1}-casein (38 %) followed by β-casein (35 %), k- and α_{s2}-casein (10-11 %), goat's milk displays a higher proportion of β-casein (55 %) and α_{s2}-casein (25 %) and a lower amount of α_{s1}-casein (5 %) [22]. The resulting peptides are in accordance with this protein composition, which indicates a balanced proteolysis of the main caseins. Peptides from *β*-casein covered almost completely the sequence, but the highest ~~abundance~~number of peptides corresponded to the (180-207) region (see Electronic supplementary material Table S1). Miclo et al. [23] reported this region as more accessible to the cell envelope protease of *S. thermophilus*. In contrast, the (15-40) region of β-casein was poorly represented in terms of matched sequences. This region comprises four phosphorylated serines and it is known that the ionization of these sequences in a complex mixture is difficult. However, the caseinophosphopeptides from β-casein ^{15}SpSpSpEESITHINK^{28}, and ^{29}KIEKFQSpEEQQQTED^{43} could be potentially identified in some samples.

It has to be highlighted that peptides from α_{s1}-casein were relatively abundant with regard to its low content in caprine milk (Electronic supplementary material Table

S2). The α_{s1}-casein structure is composed of four parts: (1) hydrophilic region (1-12), (2) hydrophobic region (13-40), (3) hydrophilic region (41-99), (4) hydrophobic region (100-199) [24]. In the present study almost all peptides were released from the hydrophobic regions, the most hydrolyzed region being that corresponding to the N-terminal hydrophobic region (22-40). Miclo et al [23] noted that the *S. thermophilus* cleavages gave rise to most peptides within the first 40 amino acid residues while the (41-99) and (152-159) regions appeared more resistant to hydrolysis and a low number of cleavage sites were observed. Accessibility to substrate was considered the determinant parameter in the protein susceptibility to hydrolysis rather than hydrophobicity. Besides, the missing peptides could derive from the phosphorylation of several residues, which impairs peptide ionization. Thus, the observed results show the contribution of different factors. No peptides from whey proteins, α-lactalbumin and β-lactoglobulin could be identified, which could be due to the low susceptibility of these compact proteins to the proteolytic action of lactic acid bacteria.

Enzymatic cleavages analysis

By the use of a sequence discrimination tool, a distinction in the generated peptides with regard to fermentation culture was revealed (Figure 1). More than 40 % of potentially identified peptides were constant in both fermented milks, which is not surprising due to the presence of the classical starter in both. However, the addition of the probiotic strain, St+LP, showed an influence on the resulting protein cleavage, with 69 exclusive sequences in this case, a similar number to the 62 exclusive fragments generated in milk fermented by the classical starter, St. In order to evaluate the hypothesis of a differential enzyme activity between cultures, a computer-assisted study of enzyme specificity was performed on the identified sequences with the EnzymePredictor program [19]. Table

12 shows the preferential enzyme cleavage rules determined for peptide sequences arisen by the action of St and St+LP. In the two best ranked enzyme specificities, no relevant differences could be found. However, the succeeding specificities were different between the fermented milks in terms of significance and a higher weight was given to the cleavage giving rise to C-terminal lysine or arginine in the case of St+LP. This cleavage specificity could be ascribed to plasmin, an endogenous enzyme in milk, responsible for the hydrolysis of α- and β-caseins. The stage of lactation affects plasmin activity and, with regard to other species, the effect in dairy goats is more pronounced since, having a seasonal breeding, they progress through lactation in a synchronous manner [25]. The higher importance of plasmin in the peptides generated by the fermented milk with St+LP could be attributed to a lower proteolytic power of the probiotic strain. This would explain this product to retain the protein cleavages present before fermentation. Therefore, although no particular enzyme could be distinctively predicted, the pattern indicated a lower proteolytic activity in St+LP milk in comparison to the classical starter bacteria, St, at least at the studied fermentation time.

Peptide bioactivity analysis

These fermented milks have previously shown ACE-inhibition, antibacterial activity against *E. coli*, and antioxidant capacity using different *in vitro* methods [9]. Peptide sequences with such reported activities or very close precursors could be found, not only with goat's milk origin but from bovine or ovine milk, due to the high sequence homology between the caseins from these dairy species. Thus, the β-casein ^{58}LVYPFTGPIPN^{68}, which has shown antihypertensive activity in spontaneously hypertensive rats [26] was found in samples fermented with the classical starter, St. The β-casein ^{195}VLGPVRGPFPI^{205}, greatly overlaps with the antihypertensive fragment

generated by *E. faecalis* on bovine milk, ^{197}VLGPVRGPFP^{206}. Other potentially identified peptides from β-casein were f(78-93), f(134-139), f(166-175), precursors of the antihypertensive sequences ^{80}TPVVVPPKLPQ^{90}, ^{134}HLPLP^{138}, and ^{169}KVLPVPQ^{175}, respectively [27], [26], [28]. Several peptides from the C-terminal β-casein region covered cow's milk sequence ^{199}VRGPFPIIV^{207}, with reported antihypertensive activity [29]. Despite this, and due to the different penultimate residue at the C-terminal sequence, leucine in goat's milk, the activity of these peptides could be different to the reported by others. Those differences between goat and cow protein sequences were previously denoted as the probable reason for the difference in ACE inhibitory activity between ^{200}GPFPI**L**V^{206} (IC50=424 μM) derived from caprine β-casein and ^{191}LLYQQPVLGPVRGPFPI**I**V^{209} (IC50=22 μM) released from bovine β-casein by hydrolysis with *Lactobacillus helveticus* CP790 proteinase [6].

Regarding other biological activities, the β-casein peptide ^{191}YQEPVLGPVRGPFPI^{205}, corresponds to casecidin 15, an antimicrobial sequence with minimal inhibition concentration against *E. coli* DPC6053 of 0.4 mg ml^{-1} [30]. On the other hand, the β-casein antioxidant peptide ^{59}VYPFTGPIPN^{68} [31] was also potentially identified. Most of these physiologically active sequences were found in the fermented milks with both cultures (see Electronic supplementary material), which supports the previously antioxidant, ACE-inhibitory and antimicrobial activities observed.

Peptide resistance to simulated digestion

Simulated digestion of fermented milks was conducted with a dialysis device intended to recover the bioaccessible fraction of peptides resistant to the digestion conditions. ~~The dialyzable fraction had significantly lower protein concentration than the other~~

~~fractions (Table 3). The comparison with the non-dialyzable fraction yields 40 % of protein dialyzable material from the total digest. Furthermore, t~~The UV-UPLC chromatographic profile of the dialyzable and non-dialyzable fractions of a digested St milk sample has been evaluated (Figure 2). The chromatographic profile does not greatly differ between fractions although an additional peak at 30 min can be observed in the non-dialyzable fraction while other peaks vary in intensity.

In the LC-MS/MS analysis a total number of 151 different peptides could be potentially identified when taken together the dialyzed and non-dialyzed fractions. From them, 72 peptides were common to St and St+ LP milks (Figure 3). The overlapping of released peptides between fermentation cultures was slightly higher after digestion (47.7 vs 43.5 %) but, still, differences in the fermented milks could be evidenced in the peptide profile, which supports the influence of the starter on the resulting digestome. The lower number of different peptides after digestion is attributed to the increase in sequence homology, as it has been previously observed [16]. Table 4 shows, from the common identified peptides, those present in the dialyzed fraction after *in vitro* digestion, because this would constitute the bioaccessible fraction. In order to know if physicochemical parameters of the sequences might determine their accumulation in the dialyzed fraction, an analysis of hydropathicity and charge of sequences was performed. The first parameter gives information about the structure of the peptide based on its hydrophobicity and hydrophilicity [32]. Notable dispersion within values was observed, with broad ranges observed for hydropathicity (-2.45 and 1.48) and charge (-2 to +2). Therefore, the peptides recovered in the dialyzed fraction could not be associated to particular features with regard to these physicochemical parameters.

On the other hand, sequence descriptors permit to anticipate the biological activity of peptides when the rationale behind their effect is defined in relation to the

amino acid chain. These sequences have been analyzed with a QSAR tool for ACE inhibition [21]. The classification model assigns the peptides to the category of potentially active (AHT) or inactive (non-AHT) in accordance to the specificity selected (Table 4). In this case the SVM score threshold was selected to provide high specificity. Positive descriptions were assigned to some sequences, with relatively high scores (over 1.7) in the case of eight sequences derived from β- and α_{s1}-casein. Some of these sequences had been reported as ACE inhibitors such as α_{s1}-casein ^{151}DAYPSGAW^{164} [33]. The identified β-casein f(81-89) displays an ample overlapping with β-casein ACE-inhibitor ^{80}TPVVVPPFLQP^{90} [27]. On the contrary, lower ranked sequences have also been described as ACE inhibitors, i. e. α_{s2}-casein ^{165}LKKISQ^{170} [34] and β-lactoglobulin ^{33}DAQSAPLRV^{41} [35].

The ACE-inhibition mechanism of action of peptides is not well known yet. In general, ACE inhibitory peptides usually contain between 2-12 amino acids [36]. Despite their activity has been linked to the C-terminal region composition and sometimes the N-terminal region influences the ACE inhibitory activity of peptides with less than six amino acid residues, the reason for the activity of higher molecular weight peptides is still unknown [37]. The presence in C-terminal position of aliphatic, aromatic or branched chain amino acids as tryptophan, tyrosine, phenylalanine, leucine, as well as proline has been considered an important feature [38],[39]. Moreover, the presence of basic amino acids such as lysine or arginine, at the C-terminal or ultimate chain position also influences this activity [40]. On the other hand, some highly ranked sequences in Table 4 have not been reported as ACE inhibitors although they fulfill these features and would merit further studies. This would be the case of sequences α_{s2}-casein ^{97}LQYPYQGPIVL^{107}, and β-casein ^{90}PEIMGVPK^{97}, and ^{157}FPPQSVL^{163}.

Regarding the antioxidant activity, the presence of the hydrophobic amino acid residues valine or leucine at the N-terminus and proline, histidine, or tyrosine in the amino acid sequence are related with antioxidant peptides, and the presence and position of tryptophan, tyrosine and methionine are thought to be responsible for the antioxidant activity [41]. In addition, casein derived peptides with glutamic and aspartic acids have been reported as able to inhibit lipid peroxidation and acid and basic amino acids played an important role in metal chelation [17]. Antibacterial activity, in turn, has been related with the peptide charge but the influence of additional physicochemical and structural properties in the mechanism of action remains to be elucidated [42].

Table 5 shows the peptides potentially found in the dialyzable fraction of St and St+LP fermented milks with reported physiological effects. Peptides with ACE inhibitory, antihypertensive, antioxidative, antibacterial, and DPP-IV inhibitory activities from the main caprine caseins and β-lactoglobulin could be found. In three cases, the determined sequence was comprised in a slightly longer active sequence. Some of these sequences are novel products from goat's milk simulated digestion, such as k-casein ^{51}INNQFLPYPY^{60}. The presence of this fragment in milk fermented with the probiotic culture *Lactobacillus plantarum* C4 after digestion deserves attention with regard to its potential DPP-IV inhibitory activity, related to the increase of lifetime of incretins. Some peptides with reported biologically activity observed before digestion were found in the non-dialyzable fractions, i. e. the antihypertensive β-casein ^{58}LVYPFTGPIPN^{68} or the antimicrobial β-casein ^{191}YQEPVLGPVRGPFPI^{205}. The dialysis might be considered an approximation to the physiological conditions of the intestinal barrier. However, the ability of the identified peptides to interact with receptors on the intestinal epithelium or to cross the mucosal barrier to exert a systemic effect should be studied to consider them as active compounds.

Conclusions

Many peptide fragments were potentially identified in the fermented milks with both cultures with a distribution according to the abundance of the parent proteins in goat's milk. Certain specificity could be assigned with regard to the different fermentation cultures with an apparently lower proteolytic activity of the probiotic strain based on the specific cleavages in the resulting peptides. The ACE-inhibitory, antioxidant and antibacterial activities previously determined in both fermented products might be attributed to the presence of peptides with these physiological effects. After simulated digestion, some of the active sequences remained but were retrieved in the non-dialyzable fraction. More importantly, digestion gave rise to active sequences able to cross the dialysis membrane. These peptides had been previously described as antihypertensive, antibacterial, antioxidative or DPP-IV inhibitors. This merits further studies on their interactions with the intestinal mucosa to assess their potential in exerting the physiological effects. Moreover, the application of QSAR analysis to the dialyzed peptide fragments after digestion allowed to designate new sequences that are candidates to be bioaccessible ACE inhibitors.

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Conflict of interest. The authors declare that they have no conflict of interest.

Figure captions

Figure 1. Venn diagrams of the peptide sequences potentially identified in the goat milk fermented with the classical starter, St, and the classical starter plus *Lactobacillus plantarum* C4, St+LP

Figure 2. UV-chromatographic profile of a) dialyzable fraction b) non-dialyzable fraction of goat's milk fermented with the classical starter, St.

Figure 3. Venn diagrams of the peptide sequences potentially identified in the goat milk fermented with the classical starter, St, and the classical starter plus *Lactobacillus plantarum* C4, St+LP after *in vitro* gastrointestinal digestion.

Table 1 Cleavage patterns computationally determined of the fermented milk peptide profile. Three amino acids upstream (P1, P2, and P3) and two downstream (P1' and P2') of the N and C -terminal cleavage sites are used.

Ranking[1]	Cleavage specificity				
	P3	P2	P1	P1'	P2'
1	Not H, K, R	Not P	Not R	F, L, W, Y	Not P
2			F, L or Y	Not P	
3			K or R		

[1]Based on odds ratio by EnzymePredictor.

H: Histidine, K: Lysine, R: Arginine, P: Proline, F: Phenylalanine, L: Leucine, W: Tryptophan, Y: Tyrosine.

Table 2. Peptides identified in fermented milks after *in vitro* digestion plus dialysis. Prediction for antihypertensive using the AHTpin platform. Support vector machine (SVM) score (threshold=0.9).

Protein	Fragment	Peptide sequence	Hydropathicity	Charge	SVM Score	Prediction
α_{s1}-casein	24 - 31	VVAPFPEV	1.31	-1	1.74	AHT
	24 - 32	VVAPFPEVF	1.48	-1	1.80	AHT
	32 - 39	FRKENINE	-1.89	0	0.01	Non-AHT
	56 - 41	DAKQMK	-1.85	1	0.09	Non-AHT
	77 - 82	EQKYIQ	-1.87	0	-0.65	Non-AHT
	110 - 114	EIVPK	-0.06	0	0.87	Non-AHT
	142 - 149	LAYFYPQL	0.56	0	1.68	AHT
	143 - 149	AYFYPQL	0.10	0	1.63	AHT
	144 - 149	YFYPQL	-0.18	0	1.11	AHT
	148 - 164	YQLDAYPSGAW	-0.54	-1	1.92	AHT
	150 - 154	FRQFY	-0.74	1	0.29	Non-AHT
	151 - 164	DAYPSGAW	-0.61	-1	1.70	AHT
	165 - 172	YYLPLGTQ	-0.15	0	-0.05	Non-AHT
	173 - 179	YTDAPSF	-0.47	-1	1.00	AHT
α_{s2}-casein	20 - 25	IYKQEK	-1.93	1	-1.06	Non-AHT
	26 - 32	NMAIHPR	-0.66	1	1.13	AHT
	90 - 96	YQKFPQY	-1.76	1	1.00	AHT
	97 - 107	LQYPYQGPIVL	0.28	0	1.35	AHT
	165 - 170	LKKISQ	-0.63	2	-0.14	Non-AHT
	165 - 171	LKKISQY	-0.73	2	-0.47	Non-AHT
	181 - 189	LKTVDQHQK	-1.58	1	-0.79	Non-AHT
	183 - 189	TVDQHQK	-2.01	0	-0.49	Non-AHT
	184 - 189	VDQHQK	-2.23	0	0.65	Non-AHT
	190 - 198	AMKPWTQPK	-1.38	2	0.39	Non-AHT
β-casein	1 - 6	REQEEL	-2.45	-2	1.00	AHT
	81 - 89	PVVVPPFLQ	1.21	0	1.92	AHT
	90 - 97	PEIMGVPK	-0.05	0	1.85	AHT
	98 - 107	VKETMVPKHK	-1.04	2	0.89	Non-AHT
	100 - 105	ETMVPK	-0.6	0	0.11	Non-AHT
	157 - 163	FPPQSVL	0.47	0	2.07	AHT
	182 - 187	DMPIQA	-0.07	-1	0.35	Non-AHT
	188 - 205	LLYQEPVLGPVRGPFPIL	0.61	0	1.38	AHT
	189 - 205	LYQEPVLGPVRGPFPIL	0.42	0	1.63	AHT
	190 - 196	LYQEPVL	0.27	-1	1.04	AHT
	190 - 205	YQEPVLGPVRGPFPIL	0.21	0	1.91	AHT
k-casein	18 - 24	FDDKIAK	-0.81	0	1.31	AHT
	42 - 48	YYQQRPV	-1.64	1	1.15	AHT
	69 - 75	SPAQTLQ	-0.64	0	-0.16	Non-AHT
	96 - 104	ARHPHPHLS	-1.39	1	0.75	Non-AHT
β-lg	32 - 41	DAQSAPLRV	-0.26	0	0.34	Non-AHT
	51 - 57	EGNLEIL	0.17	-2	-0.18	Non-AHT

β–lg: β-lactoglobulin

Table 3. Peptides with reported biological activity in the dialyzable fraction of fermented goat milks with classical starter (St), classical starter plus *Lactobacillus plantarum* C4 (St+LP) or both.

Protein	Fragment	Sequence	Activity	Reference
St and St+LP				
α_{s1}-casein	151-164	**DAYPSGAW**	ACE Inhibitor	[33]
α_{s1}-casein	144-149	**YFYPQL**	Antioxidative	[43]
α_{s2}-casein	90-96	**YQKFPQY**	Antihipertensive	[38]
α_{s2}-casein	165-170	**LKKISQ**	Antibacterial ACE Inhibitor	[44] [45]
β-casein	81-89	**PVVVPPFLQ**	ACE Inhibitor (TPVVVPPFLQP)	[27]
k -casein	96-104	**ARHPHPHLS**	Antioxidative	[46]
β-lg	33-41	**DAQSAPLRV**	ACE Inhibitor (DAQSAPLRVY)	[35]
St				
k-casein	96-105	**ARHPHPHLSF**	Antioxidative (ARHPHPHLSFM)	[46]
St + LP				
β-casein	108-113	**EMPFPK**	ACE Inhibitor	[33]
k-casein	25-30	**YIPIQY**	ACE Inhibitor Antioxidative	[47] [48]
k-casein	51-60	**INNQFLPYPY**	DPP-IV inhibitor	[13]

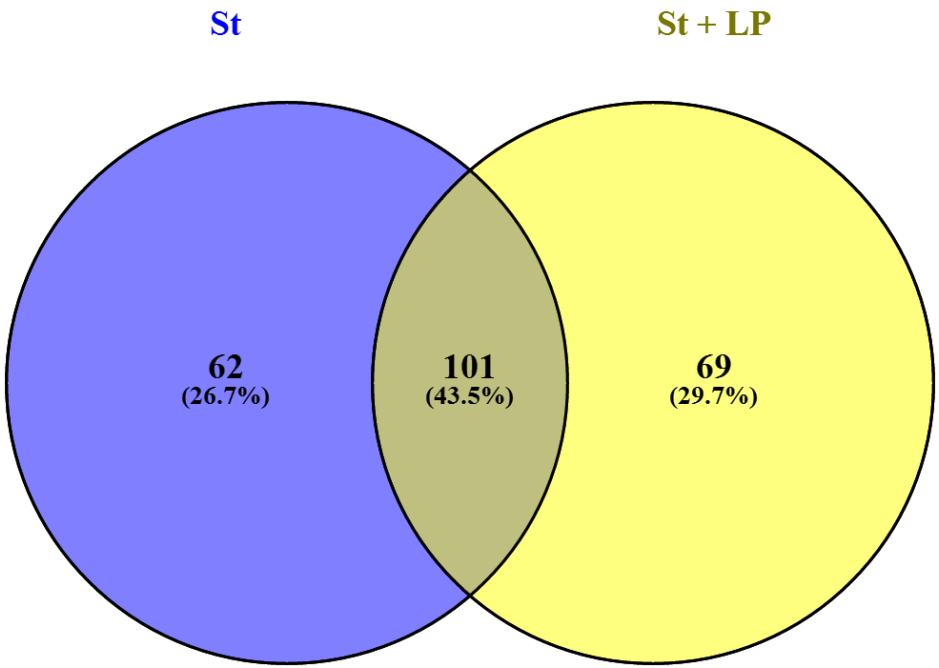
β–lg: β-lactoglobulin

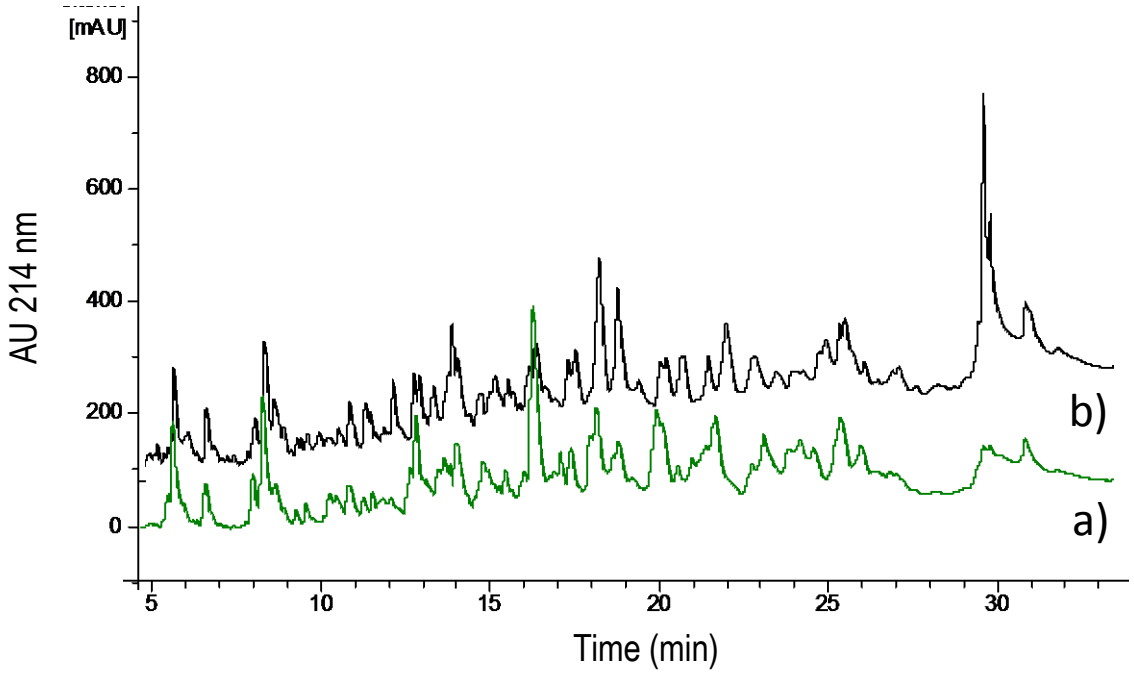
References

1. Chaves-López C, Serio A, Paparella A, Martuscelli M, Corsetti A, Tofalo R et al. Impact of microbial cultures on proteolysis and release of bioactive peptides in fermented milk. Food Microbiol. 2014;42(Supplement C):117-21. doi:https://doi.org/10.1016/j.fm.2014.03.005.
2. Liu M, Bayjanov JR, Renckens B, Nauta A, Siezen RJ. The proteolytic system of lactic acid bacteria revisited: a genomic comparison. BMC Genomics. 2010;11(1):36. doi:10.1186/1471-2164-11-36.
3. Martinez-Maqueda D, Miralles B, Recio I, Hernandez-Ledesma B. Antihypertensive peptides from food proteins: a review. Food & Function. 2012;3(4):350-61.
4. Gobbetti M, Stepaniak L, De Angelis M, Corsetti A, Di Cagno R. Latent bioactive peptides in milk proteins: Proteolytic activation and significance in dairy processing. Crit. Rev. Food Sci. Nutr. 2002;42(3):223-39. doi:10.1080/10408690290825538.
5. Minervini F, Bilancia MT, Siragusa S, Gobbetti M, Caponio F. Fermented goats milk produced with selected multiple starters as a potentially functional food. Food Microbiol. 2009;26(6):559-64. doi:https://doi.org/10.1016/j.fm.2009.03.008.
6. Quirós A, Hernández-Ledesma B, Ramos M, Amigo L, Recio I. Angiotensin-converting enzyme inhibitory activity of peptides derived from caprine Kefir. J. Dairy Sci. 2005;88(10):3480-7. doi:http://dx.doi.org/10.3168/jds.S0022-0302(05)73032-0.
7. Simsek S, Sánchez-Rivera L, El SN, Karakaya S, Recio I. Characterisation of in vitro gastrointestinal digests from low fat caprine kefir enriched with inulin. Int. Dairy J. 2017;75(Supplement C):68-74. doi:https://doi.org/10.1016/j.idairyj.2017.07.004.
8. Bergillos-Meca T, Costabile A, Walton G, Moreno-Montoro M, Ruiz-Bravo A, Ruiz-López MD. In vitro evaluation of the fermentation properties and potential probiotic activity of Lactobacillus plantarum C4 in batch culture systems. LWT-Food Sci.Technol. 2015;60(1):420-6. doi:https://doi.org/10.1016/j.lwt.2014.08.006.
9. Moreno-Montoro M, Olalla-Herrera M, Rufian-Henares JA, Martinez RG, Miralles B, Bergillos T et al. Antioxidant, ACE-inhibitory and antimicrobial activity of fermented goat milk: activity and physicochemical property relationship of the peptide components. Food & Function. 2017;8(8):2783-91. doi:10.1039/c7fo00666g.
10. Tomotake H, Okuyama R, Katagiri M, Fuzita M, Yamato M, Ota F. Comparison between Holstein cow's milk and Japanese-Saanen goat's milk in fatty acid composition, lipid digestibility and protein profile. Biosci. Biotech. Bioch. 2006;70(11):2771-4. doi:10.1271/bbb.60267.
11. Ibrahim HR, Ahmed AS, Miyata T. Novel angiotensin-converting enzyme inhibitory peptides from caseins and whey proteins of goat milk. J. Adv. Res. 2017;8(1):63-71. doi:https://doi.org/10.1016/j.jare.2016.12.002.
12. Tagliazucchi D, Shamsia S, Helal A, Conte A. Angiotensin-converting enzyme inhibitory peptides from goats' milk released by in vitro gastro-intestinal digestion. Int. Dairy J. 2017;71(Supplement C):6-16. doi:https://doi.org/10.1016/j.idairyj.2017.03.001.
13. Zhang Y, Chen R, Ma H, Chen S. Isolation and identification of dipeptidyl peptidase IV-inhibitory peptides from trypsin/chymotrypsin-treated goat milk casein hydrolysates by 2D-TLC and LC-MS/MS. J. Agric. Food Chem. 2015;63(40):8819-28. doi:10.1021/acs.jafc.5b03062.
14. Almaas H, Eriksen E, Sekse C, Comi I, Flengsrud R, Holm H et al. Antibacterial peptides derived from caprine whey proteins, by digestion with human gastrointestinal juice. Brit. J. Nutr. 2011;106(6):896-905. doi:Doi: 10.1017/s0007114511001085.
15. Ahmed AS, El-Bassiony T, Elmalt LM, Ibrahim HR. Identification of potent antioxidant bioactive peptides from goat milk proteins. Food Res. Int. 2015;74(Supplement C):80-8. doi:https://doi.org/10.1016/j.foodres.2015.04.032.
16. Sánchez-Rivera L, Diezhandino I, Gómez-Ruiz JÁ, Fresno JM, Miralles B, Recio I. Peptidomic study of Spanish blue cheese (Valdeón) and changes after simulated gastrointestinal digestion. Electrophoresis. 2014;35(11):1627-36. doi:10.1002/elps.201300510.

17. Farvin KHS, Baron CP, Nielsen NS, Otte J, Jacobsen C. Antioxidant activity of yoghurt peptides: Part 2: Characterisation of peptide fractions. Food Chem. 2010;123(4):1090-7. doi:https://doi.org/10.1016/j.foodchem.2010.05.029.
18. Bergillos-Meca T, Cabrera-Vique C, Artacho R, Moreno-Montoro M, Navarro-Alarcón M, Olalla M et al. Does Lactobacillus plantarum or ultrafiltration process improve Ca, Mg, Zn and P bioavailability from fermented goats'milk? Food Chem. 2015;187(Supplement C):314-21. doi:https://doi.org/10.1016/j.foodchem.2015.04.051.
19. Vijayakumar V, Guerrero AN, Davey N, Lebrilla CB, Shields DC, Khaldi N. EnzymePredictor: A tool for predicting and visualizing enzymatic cleavages of digested proteins. J. Proteome Res.. 2012;11(12):6056-65. doi:10.1021/pr300721f.
20. Minkiewicz P, Dziuba J, Iwaniak A, Dziuba M, Darewicz M. BIOPEP Database and Other Programs for Processing Bioactive Peptide Sequences. J. AOAC Int. 2008;91(4):965-80.
21. Kumar R, Chaudhary K, Singh Chauhan J, Nagpal G, Kumar R, Sharma M et al. An in silico platform for predicting, screening and designing of antihypertensive peptides. Scientific Reports. 2015;5:12512.
22. Tamime AY, Wszolek M, Bozanic R, Özer B. Popular ovine and caprine fermented milks. Small Ruminant Res. 2011;101(1):2-16. doi:https://doi.org/10.1016/j.smallrumres.2011.09.021.
23. Miclo L, Roux É, Genay M, Brusseaux É, Poirson C, Jameh N et al. Variability of hydrolysis of β-, α_{s1}-, and α_{s2}-Caseins by 10 strains of Streptococcus thermophilus and resulting bioactive peptides. J. Agric. Food Chem. 2012;60(2):554-65. doi:10.1021/jf202176d.
24. Kumosinski TF, Brown EM, Farrell HM, Jr. Three-dimensional molecular modeling of bovine caseins: α_{s1}-casein. J. Dairy Sci. 1991;74(9):2889-95. doi:10.3168/jds.S0022-0302(91)78470-1.
25. Fantuz F, Polidori F, Cheli F, Baldi A. Plasminogen activation system in goat milk and its relation with composition and coagulation properties. J. Dairy Sci. 2001;84(8):1786-90. doi:10.3168/jds.S0022-0302(01)74616-4.
26. Miguel M, Gómez-Ruiz J A, Recio I, Aleixandre A. Changes in arterial blood pressure after single oral administration of milk-casein-derived peptides in spontaneously hypertensive rats. Mol. Nutr. & Food Res. 2010;54(10):1422-7. doi:10.1002/mnfr.200900448.
27. Abubakar A, Saito T, Kitazawa H, Kawai Y, Itoh T. Structural analysis of new antihypertensive peptides derived from cheese whey protein by proteinase K digestion. J. Dairy Sci. 1998;81(12):3131-8. doi:10.3168/jds.S0022-0302(98)75878-3.
28. Maeno M, Yamamoto N, Takano T. Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from Lactobacillus helveticus CP790. J. Dairy Sci. 1996;79(8):1316-21. doi:http://dx.doi.org/10.3168/jds.S0022-0302(96)76487-1.
29. Quirós A, Ramos M, Muguerza B, Delgado MA, Miguel M, Aleixandre A et al. Identification of novel antihypertensive peptides in milk fermented with Enterococcus faecalis. Int. Dairy J. 2007;17(1):33-41. doi:http://dx.doi.org/10.1016/j.idairyj.2005.12.011.
30. Birkemo GA, O'Sullivan O, Ross RP, Hill C. Antimicrobial activity of two peptides casecidin 15 and 17, found naturally in bovine colostrum. J. Appl. Microbiol. 2009;106(1):233-40. doi:10.1111/j.1365-2672.2008.03996.x.
31. Eisele T, Stressler T, Kranz B, Fischer L. Bioactive peptides generated in an enzyme membrane reactor using Bacillus lentus alkaline peptidase. Eur. Food Res. Technol. 2013;236(3):483-90. doi:10.1007/s00217-012-1894-5.
32. Kyte J, Doolittle RF. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 1982;157(1):105-32. doi:http://dx.doi.org/10.1016/0022-2836(82)90515-0.
33. Pihlanto-Leppälä A, Rokka T, Korhonen H. Angiotensin I converting enzyme inhibitory peptides derived from bovine milk proteins. Int. Dairy J. 1998;8(4):325-31.
34. López-Expósito I, Gómez-Ruiz JA, Amigo L, Recio I. Identification of antibacterial peptides from ovine α_{s2}-casein. Int. Dairy J. 2006;16(9):1072-80. doi:https://doi.org/10.1016/j.idairyj.2005.10.006.
35. Tavares T, Contreras MdM, Amorim M, Pintado M, Recio I, Malcata FX. Novel whey-derived peptides with inhibitory effect against angiotensin-converting enzyme: In vitro

effect and stability to gastrointestinal enzymes. Peptides. 2011;32(5):1013-9. doi:http://dx.doi.org/10.1016/j.peptides.2011.02.005.
36. López-Fandiño R, Otte J, van Camp J. Physiological, chemical and technological aspects of milk-protein-derived peptides with antihypertensive and ACE-inhibitory activity. Int. Dairy J. 2006;16(11):1277-93. doi:http://dx.doi.org/10.1016/j.idairyj.2006.06.004.
37. FitzGerald RJ, Murray BA, Walsh DJ. Hypotensive Peptides from Milk Proteins. J. Nutr. 2004;134(4):980S-8S.
38. Contreras MdM, Carrón R, Montero MJ, Ramos M, Recio I. Novel casein-derived peptides with antihypertensive activity. Int. Dairy J. 2009;19(10):566-73.
39. Haque E, Chand R, Kapila S. Biofunctional properties of bioactive peptides of milk origin. Food Rev. Int. 2008;25(1):28-43. doi:10.1080/87559120802458198.
40. Ortiz-Chao P, Gómez-Ruiz JA, Rastall RA, Mills D, Cramer R, Pihlanto A et al. Production of novel ACE inhibitory peptides from β-lactoglobulin using Protease N Amano. Int. Dairy J. 2009;19(2):69-76. doi:http://dx.doi.org/10.1016/j.idairyj.2008.07.011.
41. Aloglu HS, Öner Z. Determination of antioxidant activity of bioactive peptide fractions obtained from yogurt. J. Dairy Sci. 2011;94(11):5305-14. doi:https://doi.org/10.3168/jds.2011-4285.
42. Demers-Mathieu V, Gauthier SF, Britten M, Fliss I, Robitaille G, Jean J. Antibacterial activity of peptides extracted from tryptic hydrolyzate of whey protein by nanofiltration. Int. Dairy J. 2013;28(2):94-101. doi:https://doi.org/10.1016/j.idairyj.2012.09.003.
43. Suetsuna K, Ukeda H, Ochi H. Isolation and characterization of free radical scavenging activities peptides derived from casein. J. Nutr. Biochem. 2000;11(3):128-31.
44. Lopez Exposito I, Minervini F, Amigo L, Recio I. Identification of antibacterial peptides from bovine kappa-casein. J. Food Protect. 2006;69(12):2992-7.
45. Lopez-Exposito I, Quiros A, Amigo L, Recio I, López Expósito I, Quirós A. Casein hydrolysates as a source of antimicrobial, antioxidant andantihypertensive peptides. Lait. 2007;87(4-5):241-9.
46. Korhonen H, Pihlanto A. Technological options for the production of health-promoting proteins and peptides derived from milk and colostrum. Curr. Pharm. Design. 2007;13(8):829-43. doi:http://dx.doi.org/10.2174/138161207780363112.
47. Gómez-Ruiz JA, Ramos M, Recio I. Identification of novel angiotensin-converting enzyme-inhibitory peptides from ovine milk proteins by CE-MS and chromatographic techniques. Electrophoresis. 2007;28(22):4202-11. doi:10.1002/elps.200700324.
48. De Gobba C, Tompa G, Otte J. Bioactive peptides from caseins released by cold active proteolytic enzymes from Arsukibacterium ikkense. Food Chem. 2014;165:205-15. doi:https://doi.org/10.1016/j.foodchem.2014.05.082.


St
St + LP
62
(26.7%)
101
(43.5%)
69
(29.7%)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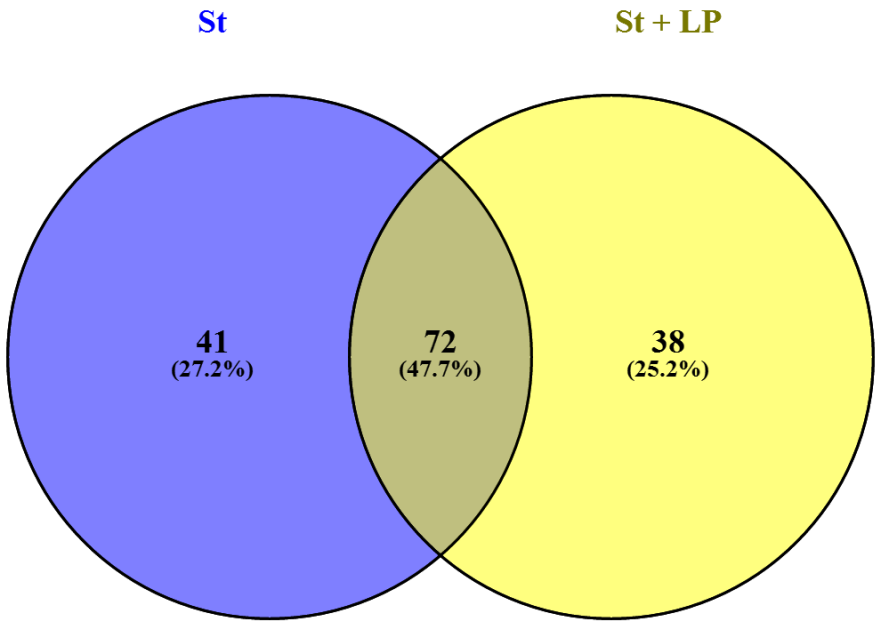

St
St + LP
41
(27.2%)
72
(47.7%)
38
(25.2%)

Analytical and Bioanalytical Chemistry

Electronic Supplementary Material

Bioaccessible peptides released by *in vitro* gastrointestinal digestion of fermented goat milks

Miriam Moreno-Montoro, Paula Jauregi, Miguel Navarro-Alarcón, Manuel Olalla-Herrera, Rafael Giménez-Martínez, Lourdes Amigo, Beatriz Miralles

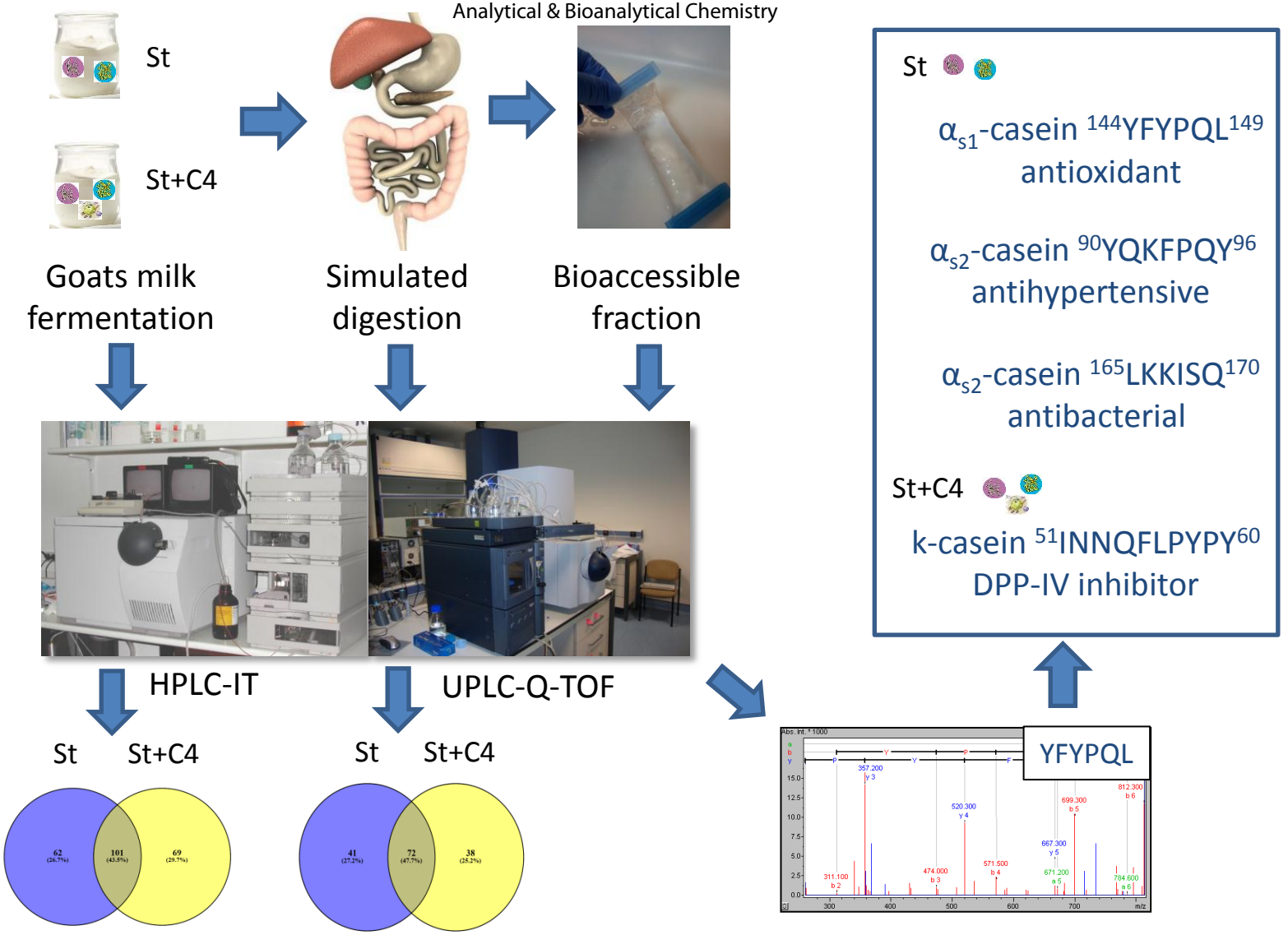
Table S1. Peptides from β-casein potentially identified in fermented milks St and St+LP

Fragment	Sequence	Observed mass	Calculated mass	Sample
1 - 5	REQEE	689.293	689.298	St
1 - 6	REQEEL	802.393	802.382	St+Lp
1 - 7	REQEELN	916.393	916.425	St
1 - 14	REQEELNVVGETVE	1629.585	1629.785	St+Lp
2 - 6	EQEEL	646.193	646.281	St
7 - 14	NVVGETVE	845.393	845.413	St
8 - 14	VVGETVE	731.293	731.370	St
14 - 18	ESpLSS	601.393	601.200	St
15 - 28	SpLSpSpSpEESITHINK	1850.385	1850.618	St
15 - 28	SLSpSpEESITHINK	1770.385	1770.652	St+Lp
17 - 28	SpSpSpEESITHINK	1570.385	1570.535	St+Lp
22 - 28	SITHINK	811.393	811.455	Both
29 - 43	KIEKFQSpEEQQQTED	1945.585	1945.831	St+Lp
41 - 46	TEDELQ	733.293	733.313	St
41 - 48	TEDELQDK	976.293	976.435	Both
44 - 52	ELQDKIHPF	1125.493	1125.582	St
48 - 56	KIHPFAQAQ	1038.493	1038.561	St+Lp
49 - 56	IHPFAQAQ	910.393	910.466	St+Lp
49 - 58	IHPFAQAQSL	1110.493	1110.582	St+Lp
50 - 56	HPFAQAQ	797.393	797.382	St
53 - 58	AQAQSL	616.393	616.318	St
57 - 68	SLVYPFTGPIPN	1303.593	1303.681	St+Lp
58 - 68	LVYPFTGPIPN	1216.593	1216.649	St
59 - 68	VYPFTGPIPN	1103.593	1103.565	Both
60 - 68	YPFTGPIPN	1004.393	1004.497	Both
62 - 68	FTGPIPN	744.393	744.381	St
63 - 70	TGPIPNSL	797.393	797.428	St
69 - 77	SLPQNILPL	993.493	993.586	St+Lp
78 - 97	TQTPVVVPPFLQPEIMGVPK	2175.985	2176.197	St+Lp
88 - 97	LQPEIMGVPK	1110.493	1110.611	St+Lp
92 - 97	IMGVPK	643.393	643.373	St
94 - 100	GVPKVKE	755.393	755.454	Both
94 - 101	GVPKVKET	856.493	856.502	St+Lp
94 - 105	GVPKVKETMVPK	1311.693	1311.758	Both
94 - 99	GVPKVK	626.393	626.412	Both
100 - 105	ETMVPK	703.393	703.357	Both
105 - 109	KHKEM	671.293	671.342	St
106 - 123	HKEMPFPKYPVEPFTESQ	2189.785	2190.046	St+Lp
108 - 123	EMPFPKYPVEPFTESQ	1924.785	1924.892	St+Lp
124 - 133	SLTLTDVEKL	1117.493	1117.623	St
126 - 133	TLTDVEKL	917.393	917.507	St

134 - 139	HLPLPL	688.393	688.427	St
134 - 141	HLPLPLVQ	915.493	915.554	Both
134 - 142	HLPLPLVQS	1002.493	1002.586	Both
134 - 143	HLPLPLVQSW	1188.593	1188.666	Both
135 - 143	LPLPLVQSW	1051.493	1051.607	St+Lp
145 - 151	HQPPQPL	815.293	815.429	St
145 - 154	HQPPQPLSPT	1100.493	1100.561	Both
155 - 163	VMFPPQSVL	1016.493	1016.536	Both
156 - 161	MFPPQS	705.393	705.316	St
157 - 163	FPPQSVL	786.393	786.428	Both
157 - 165	FPPQSVLSL	986.393	986.544	Both
164 - 180	SLSQPKVLPVPQKAVPQ	1815.185	1815.062	St
166 - 175	SQPKVLPVPQ	1091.593	1091.634	St+Lp
166 - 180	SQPKVLPVPQKAVPQ	1614.785	1614.946	Both
170 - 180	VLPVPQKAVPQ	1174.693	1174.707	St+Lp
171 - 180	LPVPQKAVPQ	1075.593	1075.639	Both
172 - 180	PVPQKAVPQ	962.493	962.555	St+Lp
173 - 180	VPQKAVPQ	865.493	865.502	Both
181 - 187	RDMPIQA	829.293	829.412	St
181 - 190	RDMPIQAFLL	1202.593	1202.648	St
182 - 189	DMPIQAFL	933.393	933.463	St
182 - 190	DMPIQAFLL	1062.393	1062.542	St
183 - 190	MPIQAFLL	931.393	931.520	St
189 - 196	LLYQEPVL	973.493	973.548	St
189 - 207	LLYQEPVLGPVRGPFPILV	2105.985	2106.224	St
190 - 205	LYQEPVLGPVRGPFPI	1780.585	1780.988	St+Lp
190 - 207	LYQEPVLGPVRGPFPILV	1992.985	1993.140	St+Lp
191 - 196	YQEPVL	747.293	747.380	St
191 - 205	YQEPVLGPVRGPFPI	1667.785	1667.904	St+Lp
191 - 207	YQEPVLGPVRGPFPILV	1879.985	1880.056	St+Lp
192 - 206	QEPVLGPVRGPFPIL	1617.585	1617.924	Both
192 - 207	QEPVLGPVRGPFPILV	1716.785	1716.993	Both
193 - 205	EPVLGPVRGPFPI	1376.693	1376.782	St
193 - 207	EPVLGPVRGPFPILV	1588.785	1588.934	St
195 - 205	VLGPVRGPFPI	1150.593	1150.686	Both
196 - 207	LGPVRGPFPILV	1263.693	1263.770	St+Lp
197 - 205	GPVRGPFPI	938.493	938.534	St+Lp
197 - 206	GPVRGPFPIL	1051.493	1051.618	St
197 - 207	GPVRGPFPILV	1150.593	1150.686	St+Lp
198 - 207	PVRGPFPILV	1093.593	1093.665	St+Lp
200 - 207	RGPFPILV	897.493	897.544	St
201 - 207	GPFPILV	741.393	741.443	St+Lp
201 - 207	GPFPILV	741.393	741.443	St+Lp
202 - 207	PFPILV	684.393	684.421	St+Lp

Table S2. Peptides from α_{s1}-casein identified in fermented milks St and St+LP

Fragment	Sequence	Observed mass	Calculated mass	Sample
17 - 24	NENLLRFV	1003.585	1003.545	St
18 - 23	ENLLRF	790.393	790.434	St
22 - 30	RFVVAPFPE	1060.493	1060.571	St
22 - 32	RFVVAPFPEVF	1306.593	1306.707	Both
22 - 35	RFVVAPFPEVFRKE	1719.785	1719.946	Both
22 - 40	RFVVAPFPEVFRKENINEL	2303.378	2303.243	Both
23 - 30	FVVAPFPE	904.393	904.469	St
23 - 31	FVVAPFPEV	1003.593	1003.538	St+LP
23 - 32	FVVAPFPEVF	1150.493	1150.606	Both
23 - 33	FVVAPFPEVFR	1306.785	1306.707	St+LP
23 - 35	FVVAPFPEVFRKE	1563.878	1563.845	Both
23 - 40	FVVAPFPEVFRKENINEL	2147.078	2147.142	Both
24 - 30	VVAPFPE	757.393	757.401	St
24 - 32	VVAPFPEVF	1003.493	1003.538	Both
24 - 35	VVAPFPEVFRKE	1416.785	1416.777	St+LP
24 - 40	VVAPFPEVFRKENINEL	2000.078	2000.073	Both
25 - 32	VAPFPEVF	904.393	904.469	Both
25 - 35	VAPFPEVFRKE	1317.785	1317.708	Both
25 - 40	VAPFPEVFRKENINEL	1901.078	1901.005	Both
26 - 32	APFPEVF	805.293	805.401	St
26 - 40	APFPEVFRKENINEL	1801.785	1801.936	St+LP
31 - 40	VFRKENINEL	1260.785	1260.683	Both
32 - 40	FRKENINEL	1161.585	1161.614	St
33 - 40	RKENINEL	1014.493	1014.546	Both
41 - 50	SKDIGSpESpTE	1211.293	1211.400	St+LP
41 - 54	SKDIGSpESpTEDQAM	1672.385	1672.558	Both
41 - 55	SKDIGSpESpTEDQAME	1801.385	1801.600	St
47 - 55	ESTEDQAME	1038.493	1038.381	St+LP
51 - 56	DQAMED	707.193	707.243	St
53 - 60	AMEDAKQM	922.293	922.389	St+LP
55 - 60	EDAKQM	736.293	736.306	Both
55 - 61	EDAKQMK	848.293	848.406	Both
56 - 60	DAKQM	591.293	591.269	St
57 - 62	AKQMKA	675.393	675.374	St
60 - 67	MKAGSSSpS	849.393	849.294	St+LP
81 - 90	IQKEDVPSER	1199.585	1199.615	St+LP
81 - 92	IQKEDVPSERYL	1475.678	1475.762	St+LP
85 - 96	DVPSERYLGYLE	1439.785	1439.693	Both
105 - 114	NVPQLEIVPK	1135.593	1135.660	Both
109 - 121	LEIVPKSAEEQLH	1490.678	1491.793	St+LP
176 - 199	APSFSDIPNPIGSENSGKTTMPLW	2545.185	2545.216	Both
185 - 199	PIGSENSGKTTMPLW	1616.785	1616.787	St+LP



Ultrafiltered goat milks were fermented with the classical starter bacteria (St) and with St plus the *L. plantarum* C4 probiotic strain. Simulated digestion was conducted in presence of a dialysis membrane to retrieve the bioaccessible peptide fraction. Samples were analyzed using HPLC-IT-MS/MS and UPLC-Q-TOF-MS/MS. Based on the specific cleavages in the resulting peptides certain specificity with regard to fermentation culture was shown. After simulated digestion, some of the active sequences remained and new peptides with reported beneficial activities were released.