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

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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 liquid chromatography-ion trap (LC-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, non-previously detected sequences 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 peptides sequences with those reported in the literature and in combination of computer-assisted prediction for ACE inhibition led to the identification of most 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 were used. Besides,he 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 number of peptides corresponded to the (180-207) region (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 potentially identified peptide 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 1

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 potentiallyidentified 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 identified peptides would probably 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 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 Kumar [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 found in the non-dialyzable fraction. More importantly, digestion gave rise to new active sequences that were able to cross the dialysis membrane . This merits further studies on their interactions with the intestinal mucosa to assess their potential in exerting the physiological effects. These peptides have been previously described as antihypertensive, antibacterial, antioxidative or DPP-IV inhibitors. 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.

Table1 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	[12]

β–lg: β-lactoglobulin

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