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Article

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Casein hydrolysates by *Lactobacillus brevis* and *Lactococcus lactis* proteases. Peptide profile discriminates strain-dependent enzyme specificity.

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Abstract

Casein from ovine and bovine milk were hydrolysed with two extracellular protease preparations from *Lactobacillus brevis* and *Lactococcus lactis*. The hydrolysates were analyzed by HPLC–MS/MS for peptide identification. A strain-dependent peptide profile could be observed, regardless of the casein origin, and the specificity of these two proteases could be computationally ascribed. The cleavage pattern yielding phenylalanine, leucine, or tyrosine at C-terminal, appeared both at *L. lactis* and *Lb. brevis* hydrolysates. However, the cleavage C-terminal to lysine was favored with *Lb. brevis* protease. The hydrolysates showed ACE-inhibitory activity with IC_{50} in the 16-70 µg/ml range. Ovine casein hydrolysates yielded greater ACE-inhibitory activity. Previously described antihypertensive and opioid peptides were found in these ovine and bovine casein hydrolysates and prediction of the antihypertensive activity of the sequences based on quantitative structure and activity relationship (QSAR) was performed. This approach might represent a useful classification tool regarding health-related properties prior to further purification.

Keywords: Lactic acid bacteria; enzyme specificity; bioactive peptides; tandem mass spectrometry; sheep milk; casein hydrolysate; QSAR

INTRODUCTION

During fermentation processes, lactic acid bacteria (LAB) hydrolyze milk proteins, mainly caseins, into peptides and amino acids which are used as nitrogen source necessary for their growth[1]. The ability of these microorganisms to produce proteolytic enzymes makes them potential producers of bioactive hydrolysates. Several strains have been reported in the literature as having an effective proteolytic system for protein hydrolysis and the release of bioactive peptides, including *Lactobacillus* strains such as *L. helveticus, L. delbrueckii ssp. bulgaricus,* and *Lactococcus* strains, *such as, L. lactis ssp. diacetylactis, L. lactis ssp. cremoris* and *Streptococcus* strains*, such as, S. thermophilus*[2, 3]. Thus, the potent antihypertensive dipeptide YP was first isolated from a yogurt-like product fermented with *L. helveticus*[4], and the extensively studied tripeptides IPP and VPP were originally found in a soft drink fermented by *L. helveticus* and *Saccharomyces cerevisiae*[5] but can be also generated by the action of *L. rhamnosus* proteinase[6]. Similarly, other casein derived peptides with ACE–inhibitory activity have been purified from fermented milk started by L*. helveticus*[7], *L. acidophilus*[8], *L. delbrueckii,* and *L. Lactis*[9]. On the contrary, *Lb. brevis* strains have not been extensively studied, and the generation of ACE-inhibitory peptides by this strain has not been reported previously. In addition to using live microorganisms, proteolytic enzymes isolated from LAB have also been successfully used in enzymatic hydrolysis processes and for the production of bioactive peptides[10]. In this way, the tripeptides IPP and VPP were produced by casein hydrolysis with *L. helveticus* CP790 extracellular proteinase[11]. In some cases, lactic fermentation is assisted by the addition of commercial enzymatic preparations[12, 13].

Arterial hypertension affects approximately 25% of the adult population worldwide and is predicted to reach 29% of the population by 2025[14, 15]. Due to this high prevalence, food components, such as food peptides, with lowering blood pressure properties are receiving special attention. Angiotensin converting enzyme (ACE, EC3.4.15.1) plays an important role in the regulation of arterial pressure because it catalyzes the conversion of angiotensin-I (the inactive form) to angiotensin-II (a vasoconstrictor) and inactivates bradykinin (a vasodilator). On the other hand, opioid receptors have been shown to be involved in the antihypertensive effect of some food peptides. Nurminen and co-workers[16] demonstrated this relationship in the antihypertensive effect of α-lactorphin, through the endothelium-dependent relaxation of the mesenteric arteries in spontaneous hypertensive rats. The mechanism of action was driven by the stimulation of peripheral opioid receptors and subsequent nitric oxide release, causing vasodilatation. This places the ACE-inhibitor and opioid ligands as target peptides in the search of responsible agents for the antihypertensive effects of food hydrolysates.

The objective of this study was to obtain casein hydrolysates of bovine and sheep milk with proteases preparations from selected strains from *Lb. brevis* and *L. lactis*, and to investigate if the released peptide sequences could be ascribed to a cleavage pattern, characteristic of the LAB origin. In addition, the ACE-inhibitory activity of the hydrolysates was determined and QSAR analysis was applied to evaluate the occurrence of promising antihypertensive peptide sequences.

MATERIAL AND METHODS

Preparation of casein

Ovine casein was prepared from a collected sheep milk sample from the region of Hassi R'mel-Laghouat (Algeria) by acid precipitation[17]. Fresh milk was previously defatted by centrifugation (8000 × *g* for 20 min at 4°C) and lipid layer removal, repeating the process twice. The whole casein was precipitated from skimmed fresh milk with 1 N HCl at pH 4.6 and 30°C followed by heating to 35°C, and holding for 10 min. The precipitate was recovered by centrifugation at 5000 × *g* for 10 min and washed twice with water (pH maintained at 4.6), dissolved at pH 7.0 with 1 N NaOH, re-precipitated and washed. The casein was then lyophilized and stored at - 20°C. Commercial bovine casein was purchased from Prolabo (Fontenay-sous-Bois, France).

Bacterial strains and growth conditions

Two strains of lactic acid bacteria previously selected as proteolytic strains that belong to the collection of Laboratoire de Biologie des Microorganismes et Biotechnologie, LBMB. Université d'Oran 1 Ahmed Ben Bella (Oran, Algérie) were used in this study: *Lb. brevis* was isolated from camel milk[18] and *L. lactis* from cow milk. The LAB strains were maintained in reconstituted skimmed milk (11%) at -20°C. Standard cultures were prepared by inoculation of 10 ml MRS broth[19] at pH 5.7 and M17 broth[20] at pH 7.2 with the frozen stocks followed by incubation at 30°C for 24 h.

Extracellular proteases preparation

The MRS and M17 broth medium buffered (KH_2PO_4/Na_2HPO_4 0.1M pH 7) and supplemented with 2% skimmed milk were inoculated at 2% by pre-cultured strains (DO_{600nm}=1), and incubated at 30°C for 48 h in an orbital shaker (125 rpm). The cultures were centrifuged (6000 × *g* for 10 min at 4°C), to recover the supernatants containing the extracellular proteases. The proteases were precipitated from culture supernatants by the gradual addition of solid ammonium sulfate to achieve 80% saturation at 4°C; this mixture was allowed to precipitate under constant agitation for 3 h, centrifuged (8000 × *g* for 20 min at 4°C), and the resulting pellet was dissolved in a Tris-HCl buffer 0.1 M pH 7. The concentrated enzyme samples were dialyzed against water (pore size 1,5-3 nm). The proteolytic activity was evaluated by the agar diffusion assay by using skim milk as substrate. The medium contained agar and reconstituted skim milk at a final concentration of 1% in the presence of NaN_3 (0.02%). After incubation at 30°C for 48 h, the proteolysis of milk casein was revealed by the presence of a clear halo around the well. Fractions showing proteolytic activity were pooled to prepare the ovine and bovine casein hydrolysates.

Preparation of casein hydrolysates

Casein solutions (2.5% in 0.1 N NaOH and adjusted to pH 7.8) were heated at 90°C for 15 min and then sterilized by autoclaving at 110°C for 15 min. To hydrolyze the caseins, the reaction mixture contained one volume of the enzyme preparation, one volume of the casein solution and one volume of the Tris-HCl buffer 0.1 M pH 7. The mixture was incubated at 37°C in an orbital shaker (150 rpm) for 48 h, the reaction was stopped by

heating at 80°C for 15 min. The hydrolysates were centrifuged (6000 × *g* for 15 min) and the supernatants were collected. The protein concentration of supernatants was determined by the bicinchoninic acid method using bovine serum albumin as a standard[21]. The hydrolysates were passed through ultrafiltration membranes with a cut-off of 5 kDa. The filtrates were then freeze-dried and stored at −20°C until reconstitution with distilled water for *in vitro* ACE-inhibitory assay and peptide analysis.

***In vitro* assay of ACE-inhibitory effect**

ACE-inhibitory activity was measured by a fluorometric assay[22], with some modifications[23]. The angiotensin converting enzyme (ACE, peptidyl-dipeptidase A, EC3.4.15.1) was obtained from Sigma-Aldrich (St. Louis, MO, USA) and the ACE working solution was added to blank (B), control (C) or samples (S). The reaction was started by adding the fluorogenic substrate o-aminobenzoylglycyl-p-nitro-L-phenylalanyl-L-proline (Abz-Gly-p-Phe (NO2)-Pro-OH) (0.45 mM, Bachem Feinchemikalien, Bubendorf, Switzerland). Ninety six-well microplates were used and the reaction mixture was incubated at 37°C. The fluorescence of the samples was measured in a Multiscan Microplate Fluorimeter (FLUOstar optima, BMG Labtech, Offenburg, Germany), with the FLUOstar (version 1.32 R2, BMG Labtech) control system for data processing.

The activity of each sample was tested in triplicate. ACE-inhibitory activity was expressed as the protein concentration required to inhibit 50% the initial ACE activity (IC_{50}). The percentage of ACE-inhibitory activity was calculated as: 100 × (C − S)/(C − B). This parameter was plotted versus protein concentration and non-linear adjustment

was performed to estimate the IC_{50} values with the Prism 4 for Windows software (GraphPad Software, Inc. San Diego, CA, USA), as previously described by Quirós et al.[23]

Peptide analysis by reversed phase-high-performance liquid chromatography tandem mass spectrometry (RP-HPLC-MS/MS)

The analyses by RP-HPLC-MS/MS 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[24]. Chromatographic separations were performed with a Mediterranea Sea_{18} 150 mm × 2.1 mm column (Teknokroma, Barcelona, Spain). Samples were injected at a protein concentration of 3 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. Peptide sequencing was performed by MASCOT (matrixscience.com), using a homemade database that included the main bovine and ovine milk proteins. Error tolerances used were 0.1% for precursor masses and 0.5 Da for fragment masses. The matched MS/MS spectra were interpreted by using BioTools version 3.2 (Bruker). Venn's diagrams were performed with Venny 2.1 (http://bioinfogp.cnb.csic.es/tools/venny/index.html). Peptide sequences were analysed by the Enzyme Predictor tool[25]. The AHT pin *in silico* platform (http://crdd.osdd.net/raghava/ahtpin/#)[26] was used in the variable length mode, amino acid composition model, and the SVM threshold was set to 0.9.

RESULTS AND DISCUSSION

Enzymatic specificity in the hydrolysates peptide profile

The extracellular protease preparations from the selected *Lb. brevis* and *L. lactis* strains were shown to be suitable to hydrolyze casein due to their notable proteolytic activity, as shown by skim milk diffusion assay (results not shown). The major peptide components of each hydrolysate were identified by RP-HPLC-MS/MS, and these results are summarized in Tables 1 and 2 for ovine casein, and Tables 3 and 4 for bovine casein. As expected, most fragments derived from the most abundant casein fractions, i.e., β-casein, α_{s1}-casein, while a lower number of peptides were from α_{s2}- and κ-casein peptides. A slightly greater number of peptides were found in the ovine than in bovine casein hydrolysates. This better performance of the ovine casein product could be due to this casein was isolated at the laboratory while the bovine casein was a commercial product. This effect had been previously observed when studying the peptidome generated through simulated gastric digestion of heated and non-heated milk when better protein coverage was obtained for the non-heated sample[27]. This could be partly attributed to changes promoted during heat treatment, such as, lactosylation amino acid racemization or protein-protein interaction.

The number of amino acids of the identified peptides ranged from 5 to 17 residues. The longest sequences (over 15 amino acids) corresponded to the C-terminal region of β-casein and were only found in the ovine casein hydrolysates (Tables 1 and 2). On the contrary, short sequences (less than 8 residues) could be found in all hydrolysates. In addition, it could be observed certain selectivity with the bacterial extract used. To

illustrate this point, Figure 1 shows the resulting Venn diagrams from the comparison of the total number of identified peptides, with regard to the bacterial origin of the protease extract. A small overlapping of sequences is evident, as it represents 8% of the total number of peptides. This result points to a different proteolytic behavior for the *L. lactis* and *Lb. brevis* preparations, regardless of the milk casein origin, ovine or bovine. Differences in the generated peptides could be attributed to genomic differences among *L. lactis* and *Lb. brevis*. By genome comparative hybridization it has been shown proteolytic diversity between both species at the level of endopeptidases PepE/PepG with proline peptidases PepI and PepR only present in *Lb. brevis* species[28]. Additional differences in some dipeptidases were found, not only between both species, but also concerning *Lactobacillus* subspecies[28].

In order to determine, from the peptide products, certain cleavage specificities, we have employed the EnzymePredictor tool[25]. Table 5 shows the most likely cleavage specificity computationally determined for the hydrolysates, following the nomenclature of enzymatic cleavage model created by Schechter and Berger[29]. The cleavage pattern yielding phenylalanine, leucine, or tyrosine at C-terminal, appeared both at *L. lactis* and *Lb. brevis* hydrolysates. However, while the positively charged residues lysine and arginine could appear at the C-terminal in the *L. lactis* hydrolysates, only the cleavage C-terminal to lysine is favored with *Lb. brevis* protease. In addition, the cleavages with *Lb. brevis* seems to be more restricted regarding the position P1´, being preferred the cleavages N-terminal to leucine and aromatic residues (tyrosine, phenylalanine and tryptophan). Following the pattern shown in Table 5, from the

peptides generated by the *L. lactis* preparation, 86% (i.e., 61 out of 71) were correctly ascribed and 74% in the case of *Lb. brevis*.

It is worth to highlight that the genetic variations in N- and C-termini flanking residues according to the casein species, ovine or bovine, can also determine certain specificity. The cleavage β-casein 132-133 was observed in the hydrolysates prepared with *Lb. brevis* with both, ovine and bovine casein, but the hydrolysis of this bond was only observed with *L. lactis* with ovine casein (Figure 2). These results agree with the specificity shown in Table 5 where this bond could be hydrolysed with *Lb. brevis* regardless of the species. However, the release of the ^{133}leucine can be only expected in the *L. lactis* hydrolysate of ovine casein, due to the preceding ^{132}lysine, while in bovine casein, the preceding ^{132}asparagine does not favor its cleavage.

In LAB, it is generally recognized that the cell-wall anchored protease PrtP is responsible for casein degradation outside the cell. However, attempts to identify surface-located peptidases capable to cleave further casein-derived peptides released by PrtP have not been successful[3]. A peptidomic approach with the aim to analyze the cell-surface proteolytic processing in *L. lactis* has been recently performed. Analysis of the generated peptides permitted to demonstrate the presence of new proteolytic activities at the surface of the bacterium[30]. The information about enzyme specificity provided by this analysis might be advantageous for the food industry, because it means that the bacterium could be replaced by mixtures of food grade enzymes with the same enzyme specificity.

ACE inhibitory activity of the hydrolysates

The hydrolysates (fractions lower than 5 kDa) were assayed for ACE-inhibitory activity. The IC_{50} values are shown in Table 6. The ovine casein hydrolysates exhibited greater ACE-inhibitory activity, i.e., lower IC_{50} values, probably due to the hydrolysis into small size peptides, as above mentioned. These IC_{50} values were comparable to those reported for other protein hydrolysates by fermentation or enzymatic digestion. Thus, milk fermented with *Enteroccocus faecalis* presented IC_{50} values in the 34-59 μg/ml range, depending on the strain[31] and casein hydrolysed with pepsin showed IC_{50} values in the 25-60 μg/ml range, depending on the hydrolysis time[32]. Fermentation with *L. delbrueckii* and *S. thermophilus*, in combination with previous enzymatic hydrolysis, was used to produce fermented whey beverages with similar IC_{50} values[33]. Some of these fermentation/hydrolysis products have further shown antihypertensive activity in spontaneously hypertensive rats.

The sequences found in the hydrolysates have been analyzed with the QSAR platform AHTpin developed to predict antihypertensive peptides. This tool has been selected because it takes into account peptides with a broad size range (from 2 to >12 amino acid residues). The inclusion of a negative dataset, i.e., sequences displaying no bioactivity, permits the classification model to assign peptides to the category of potentially active or inactive[26]. The rationale behind the activity of specific compounds in relation with their molecular or physicochemical descriptors constitutes the basic principle of QSAR. In general, the C-terminal sequence of the peptide appears as the major contributor to the activity. The presence of aliphatic hydrophobic and small amino acids (alanine, tryptophan, proline, phenylalanine, glycine, cysteine, leucine and isoleucine) at the C_1 position of peptides is a good predictor for potent *in vitro* ACE

inhibitory activity. Besides, the nature of C_2 to C_4 amino acids modulates the inhibitory potential[34]. This arises by the specific mode of action of the enzyme, a dipeptidyl carboxypeptidase, where peptide binding to the active site occurs through the C-terminal dipeptide sequence, with a positive contribution of hydrophobicity to the binding[35].

In all hydrolysates, an important number of peptides with predicted antihypertensive activity were found (Tables 1-4). In the ovine casein hydrolysate with the *Lb. brevis* preparation, high SVM scores were found for sequences where the C-terminal tripeptide included proline, at C_1, i.e., β-casein ^{133}LHLPLP^{138}, ^{189}LLYQEPVLGP^{198}, ^{190}LYQEPVLGP^{198}, ^{191}YQEPVLGP^{198}; C_2, i.e., β-casein ^{62}FTGPIPN^{68}, ^{165}LSQPK^{169}, ^{199}VRGPFPI^{205}, α_{s1}-casein ^{23}FVVAPFPE^{30}, ^{24}VVAPFPE^{30}, α_{s2}-casein ^{101}YQGPIVLNPW^{106}, ^{114}YPVEPF^{119}, κ-casein ^{55}FLPYPY^{66} ; or C_3, i.e., β-casein ^{155}VMFPPQS^{161}, and α_{s2}-casein ^{90}YQKFPQY^{96} . The neighbor residues met to a high extent the above mentioned size and chain-structure features, being glycine, isoleucine, phenylalanine and tryptophan. The location of an hydrophobic amino acid flanked by proline residues close to the C-terminal, such as, in the α_{s1}-casein fragment ^{23}FVVAPFP^{29}, generated also a high score. This structure with proline at the C-terminal and antepenultimate positions and an aromatic residue as penultimate residue has been reported as important for ACE-inhibition[36]. Besides, peptides displaying the amino acid motif proline-hydrophobic-proline have shown resistance to further proteolytic degradation[37]. In the bovine casein hydrolysate with *L. lactis*, the best ranked sequences displayed the dipeptide valine-phenylalanine in C_2-C_1 or C_3-C_2, such as in α_{s1}-casein fragments ^{25}VAPFPEVF^{32}, ^{26}APFPEVF^{32}, and ^{25}VAPFPEVFG^{33}. These sequences are comprised in the

antihypertensive peptide α_{s1}-casein ^{23}FFVAPFPEVFGK^{34} described by Karaki et al., (1990)[38].

Table 7 shows the identified peptides in these ovine and bovine casein hydrolysates that had previously been described as antihypertensive or ACE-inhibitors and their origin with regard to bacterial preparation. The β-casein fragments ^{133}LHLPLPL^{139}, ^{133}LHLPLP^{138} and the α_{s2}-casein ^{90}YQKFPQY^{96} have shown antihypertensive effect on spontaneously hypertensive rats[23, 32, 39]. In the casein hydrolysates with the *L. lactis* protease, phenylalanine prevailed as C-terminal amino acid in peptides with positive prediction, such as, α_{s1}-casein ^{23}FVVAPFPEVF^{32} and β-casein ^{113}KYPVEPF^{119}, and ^{114}YPVEPF^{119}. The latter corresponds to the opioid sequence neocasomorphin[40]. Peptides with tryptophan in C_2, i.e., α_{s1}-casein ^{155}QLDAYPSGAWY^{165} and ^{156}LDAYPSGAWY^{165}, were notably ranked and the comprised α_{s1}-casein fragment ^{157}DAYPSGAW^{164} has shown ACE-inhibitory activity (Table 7). Moreover, the tripeptide, ^{162}GAW^{164}, has shown potent antihypertensive activity in SHR[12], which indicates that further cleavage of the peptide might release the antihypertensive form.

For some of the identified sequences with no previous reports of antihypertensive effect, experiments to assay this activity could be advised. The elevated number of positively predicted sequences would be consistent with the low IC_{50} values determined. Nevertheless, peptide concentration, and, in some cases, synergism with other compounds in the hydrolysate will determine the final effect.

In accordance with the results presented in this study, casein from ovine or bovine milk could be a suitable substrate for hydrolysis by extracellular protease preparations

from *Lb. brevis* and *L. lactis*. The cleavage pattern analysis has permitted to show a strain-dependent peptide profile and to computationally ascribe the most probable cleavage specificities. The cleavage pattern yielding phenylalanine, leucine, or tyrosine at C-terminal, appeared both at *L. lactis* and *Lb. brevis* hydrolysates. However, the positively charged residues lysine and arginine could appear at the C-terminal in the *L. lactis* hydrolysates, while the cleavage C-terminal to lysine is favored with *Lb. brevis* protease. This hydrolysis generates peptides previously reported with ACE-inhibitory, antihypertensive and opioid activities. Hence, the generation of antihypertensive peptides by using *Lb. brevis* had not been previously reported. The QSAR predictions for antihypertensive sequences have revealed promising sequences that would merit antihypertensive activity assays. This computational information could be used to select enzymatic preparations suitable for production of health beneficial hydrolysates.

ABBREVIATIONS: ACE, angiotensin I-converting enzyme; LAB, lactic acid bacteria; QSAR, quantitative structure and activity relationship; RP-HPLC-MS/MS, reversed phase-high-performance liquid chromatography-tandem mass spectrometry.

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FIGURE CAPTIONS

Figure 1. Venn diagrams of the peptide sequences identified in the ovine and bovine casein hydrolysates with regard to the extracellular preparation origin.

Figure 2. Comparative alignment of bovine (grey) and ovine (red) β-casein identified peptides in the casein hydrolysates generated with extracellular proteases from *Lactobacillus brevis* and *Lactococcus lactis*. Peptides generated from bovine casein by *L. lactis* are shown in grey and those produced by *Lb. brevis* are shown in light grey. Peptides generated from ovine casein by *L. lactis* are shown in red and those produced by *Lb. brevis* are shown in light red.

Table 1: Identification of peptides in sheep casein hydrolysates produced by partially purified proteinases of *Lb. brevis*. Support vector machine (SVM) score (threshold=0.9) and *in silico* prediction for antihypertensive using the AHTpin platform.

Observed mass	Calculated mass	Protein	Fragment	Sequence	SVM Score	Prediction
746.3	746.345	β-casein	43 - 48	DELQDK	-0.19	Non-AHT
631.2	631.318		44 - 48	ELQDK	0.38	Non-AHT
744.2	744.381		62 - 68	FTGPIPN	1.41	AHT
556.9	557.281		68 - 72	NSLPQ	0.64	Non-AHT
1196.4	1196.681		77 - 87	LTQTPVVVPPF	1	AHT
1083.4	1083.596		78 - 87	TQTPVVVPPF	0.97	Non-AHT
656.2	656.390		82 - 87	VVVPPF	1	AHT
747.3	747.363		108 - 113	EMPFPK	1	AHT
750.2	750.359		114 - 119	YPVEPF	1.43	AHT
816.3	816.459		127 - 133	LTDVEKL	0.02	Non-AHT
688.3	688.427		133 - 138	LHLPLP	1.13	AHT
1427.4	1427.702		143 - 154	WMHQPPQPLPPT	0.88	Non-AHT
804.2	804.384		155 - 161	VMFPPQS	1.66	AHT
870.3	870.517		162 - 169	VLSLSQPK	0.42	Non-AHT
571.0	571.333		165 - 169	LSQPK	1.23	AHT
779.3	779.491		170 - 176	VLPVPQK	1.7	AHT
761.2	761.396		189 - 194	LLYQEP	1	AHT
1127.4	1127.623		189 - 198	LLYQEPVLGP	1.35	AHT
1780.8	1780.988		189 - 204	LLYQEPVLGPVRGPFP	2.06	AHT
1014.4	1014.539		190 - 198	LYQEPVLGP	1.56	AHT
901.3	901.455		191 - 198	YQEPVLGP	1.71	AHT
784.3	784.460		199 - 205	VRGPFPI	1.66	AHT
996.4	996.612		199 - 207	VRGPFPILV	0.48	Non-AHT
741.3	741.443		201 - 207	GPFPILV	1.35	AHT
770.2	770.417	α_{s1}-casein	11 - 17	LSPEVLN	0.93	Non-AHT
1013.3	1013.503		11 - 19	LSPEVLNEN	1.12	AHT
904.3	904.469		23 - 30	FVVAPFPE	2.02	AHT
757.3	757.401		24 - 30	VVAPFPE	2.14	AHT
677.3	677.386		31 - 35	VFRKE	0.03	Non-AHT
734.2	734.345		41 - 47	SKDIGSE	-1.22	Non-AHT
701.2	701.334		85 - 90	DVPSER	-0.65	Non-AHT
853.3	853.437		164 - 169	WYYLPL	1.1	AHT
652.2	652.270		173 - 178	YTDAPS	-0.39	Non-AHT
886.2	886.371		173 - 180	YTDAPSFS	0.8	Non-AHT
723.1	723.308		174 - 180	TDAPSFS	0.04	Non-AHT
940.3	940.450		181 - 189	DIPNPIGSE	0.34	Non-AHT
1054.3	1054.493		181 - 190	DIPNPIGSEN	0.48	Non-AHT
972.3	972.471	α_{s2}-casein	90 - 96	YQKFPQY	1	AHT

1298.5	1298.702		100 - 110	LYQGPIVLNPW	0.9	Non-AHT
1185.4	1185.618		101 - 110	YQGPIVLNPW	1.23	AHT
961.3	961.459	κ-casein	55 - 61	FLPYPYY	1.17	AHT
741.2	741.345		34 - 39	RYPSYG	1	AHT
798.2	798.395		55 - 60	FLPYPY	1.45	AHT

Table 2: Identification of peptides in sheep casein hydrolysates produced by partially purified proteinases of *L. lactis.* Support vector machine (SVM) score (threshold=0.9) and *in silico* prediction for antihypertensive using the AHTpin platform.

Observed mass	Calculated mass	Protein	Fragment	Sequence	SVM Score	Prediction
724.1	724.380	β-casein	57 - 62	SLVYPF	0.84	Non-AHT
744.1	744.381		62 - 68	FTGPIPN	1.41	AHT
930.4	930.521		98 - 105	VKETMVPK	1	AHT
703.2	703.357		100 - 105	ETMVPK	0.11	Non-AHT
747.2	747.363		108 - 113	EMPFPK	1	AHT
878.3	878.454		113 - 119	KYPVEPF	1.48	AHT
750.2	750.359		114 - 119	YPVEPF	1.43	AHT
801.4	801.511		133 - 139	LHLPLPL	1.18	AHT
649.2	649.289		140 - 144	VQSWM	-0.28	Non-AHT
779.3	779.491		170 - 176	VLPVPQK	1.7	AHT
820.2	820.379		182 - 188	DMPIQAF	0.72	Non-AHT
1893.6	1894.072		189 - 205	LLYQEPVLGPVRGPFPI	1.63	AHT
1880.4	1880.056		191 - 207	YQEPVLGPVRGPFPILV	1.83	AHT
1716.6	1716.993		192 - 207	QEPVLGPVRGPFPILV	1.7	AHT
865.3	865.502		193 - 200	EPVLGPVR	1.03	AHT
741.3	741.443		201 - 207	GPFPILV	1.35	AHT
757,2	757.408	α_{s1}-casein	17 - 22	NENLLR	0.97	Non-AHT
775,3	775.427		23 - 29	FVVAPFP	1.62	AHT
904,3	904.469		23 - 30	FVVAPFPE	2.02	AHT
1150,4	1150.606		23 - 32	FVVAPFPEVF	1.71	AHT
945,3	945.477		35 - 42	ENINELSK	0.04	Non-AHT
663,2	663.290		53 - 58	AMEDAK	-0.33	Non-AHT
830,3	830.377		84 - 90	EDVPSER	0.98	Non-AHT
884,3	884.428		91 - 97	YLGYLEQ	-0.26	Non-AHT
860,3	860.476		103 - 109	KYNVPQL	1.36	AHT
922,3	922.549		107 - 114	PQLEIVPK	1.3	AHT
813,2	813.406		145 - 150	FYPQLF	0.31	Non-AHT
822,3	822.439		146 - 151	YPQLFR	0.68	Non-AHT
792,2	792.365		155 - 161	QLDAYPS	2.02	AHT
1269,3	1269.567		155 - 165	QLDAYPSGAWY	1.92	AHT
1141,3	1141.508		156 - 165	LDAYPSGAWY	2.3	AHT
953,3	953.486		166 - 173	YLPLGTQY	-0.05	Non-AHT
790,2	790.423		167 - 173	LPLGTQY	0.14	Non-AHT
759,3	759.399		194 - 199	ITMPLW	0.51	Non-AHT
1073,5	1073.524	α_{s2}-casein	7 - 16	VSSSEEPINI	-0.68	Non-AHT
947,2	947.471		82 - 89	ALNEINQF	1.05	AHT
666,2	666.338		93 - 97	FPQYL	1.39	AHT
928,3	928.465		168 - 174	ISQYYQK	0.51	Non-AHT

810,2	810.370		175 - 180	FAWPQY	1.21	AHT
677,2	677.338		199 - 204	TNAIPY	0.92	Non-AHT
795.2	795.417	κ-casein	25 - 30	YIPIQY	1	AHT

Table 3: Identification of peptides in the bovine casein hydrolysate produced by partially purified proteinases of *Lb. brevis*. Support vector machine (SVM) score (threshold=0.9) and *in silico* prediction for antihypertensive using the AHTpin platform.

Observed mass	Calculated mass	Protein	Fragment	Sequence	SVM Score	Prediction
787.4	787.408	β-casein	1 - 6	RELEEL	1	AHT
1212.6	1212.654		58 - 68	LVYPFPGPIPN	2.13	AHT
1098.9	1099.57		59 - 68	VYPFPGPIPN	2.32	AHT
772.2	772.379		88 - 94	LQPEVMG	1	AHT
801.5	801.511		133 - 139	LHLPLPL	1.18	AHT
804.4	804.384		155 - 161	VMFPPQS	1.66	AHT
1103.6	1103.569		155 - 164	VMFPPQSVLS	1.26	AHT
907.6	907.585		169 - 176	KVLPVPQK	1.63	AHT
1002.4	1002.459		179 - 186	PYPQRDMP	0.39	Non-AHT
742.2	742.343		181 - 186	PQRDMP	1	AHT
761.2	761.396		191 - 196	LLYQEP	1	AHT
1127.6	1127.623		191 - 200	LLYQEPVLGP	1.35	AHT
648.1	648.312		192 - 196	LYQEP	1.56	AHT
1014.5	1014.539		192 - 200	LYQEPVLGP	1.56	AHT
868.5	868.465	α_{s1}-casein	10 - 17	GLPQEVLN	0.12	Non-AHT
700.2	700.376		15 - 20	VLNENL	0.81	Non-AHT
805.3	805.401		24 - 30	FVAPFPE	1.59	AHT
706.2	706.401		31 - 36	VFGKEK	0.61	Non-AHT
864.4	864.398		85 - 91	DVPSERY	1	AHT
761.2	761.48		102 - 107	KKYKVP	-0.35	Non-AHT
761.3	761.444		103 - 108	KYKVPQ	0.45	Non-AHT
978.4	978.445		156 - 164	LDAYPSGAW	2.18	AHT
865.3	865.361		157 - 164	DAYPSGAW	1.7	AHT
1040.5	1040.493	κ-casein	31 - 39	VLSRYPSYG	0.65	Non-AHT
941.5	941.493		32 - 39	LSRYPSYG	0.41	Non-AHT
741.2	741.193		34 - 39	RYPSYG	1	AHT
825.2	825.193		42 - 47	YYQQKP	0.25	Non-AHT
961.4	961.393		55 - 61	FLPYPYY	1.17	AHT
798.3	798.293		55 - 60	FLPYPY	1.45	AHT
727.3	727.293		66 - 72	AVRSPAQ	-0.62	Non-AHT

Table 4: Identification of peptides in bovine casein hydrolysates produced by partially purified proteinases of *L. lactis*. Support vector machine (SVM) score (threshold=0.9) and *in silico* prediction for antihypertensive using the AHTpin platform.

Observed mass	Calculated mass	Protein	Fragment	Sequence	SVM Score	Prediction
1035.4	1035.535	β-casein	64 - 73	GPIPNSLPQN	1.44	AHT
552.0	552.327		73 - 77	NIPPL	1.18	AHT
854.3	854.490		80 - 87	TPVVVPPF	1.47	AHT
967.4	967.574		80 - 88	TPVVVPPFL	1.45	AHT
936.3	936.485		103 - 110	APKHKEMP	0	Non-AHT
878.2	878.436		109 - 115	MPFPKYP	0.29	Non-AHT
975.3	975.507		111 - 118	FPKYPVEP	1.58	AHT
731.1	731.385		113 - 118	KYPVEP	1.49	AHT
603.1	603.290		114 - 118	YPVEP	1.54	AHT
697.2	697.292		119 - 124	FTESQS	0.59	Non-AHT
651.2	651.359		149 - 154	QPLPPT	1.18	AHT
804.3	804.384		155 - 161	VMFPPQS	1.66	AHT
779.3	779.491		170 - 176	VLPVPQK	1.7	AHT
741.3	741.443		203 - 209	GPFPIIV	1.53	AHT
805.2	805.401	α_{s1}-casein	24 - 30	FVAPFPE	1.59	AHT
658.2	658.333		25 - 30	VAPFPE	0.92	AHT
904.3	904.469		25 - 32	VAPFPEVF	2.02	AHT
961.3	961.491		25 - 33	VAPFPEVFG	1.84	AHT
805.2	805.401		26 - 32	APFPEVF	1.59	AHT
637.2	637.311		28 - 32	FPEVF	0.04	Non-AHT
949.4	949.487		32 - 39	FGKEKVNE	-1.5	Non-AHT
834.2	834.449		92 - 98	LGYLEQL	-0.69	Non-AHT
830.2	830.385		144 - 149	YFYPEL	1	AHT
792.2	792.365		156 - 163	LDAYPSGA	1.58	AHT
745.2	745.307		161 - 166	SGAWYY	0.64	AHT
776.2	776.407		167 - 173	VPLGTQY	0.73	Non-AHT
788.2	788.370		179 - 185	FSDIPNP	0.51	Non-AHT
729.2	729.329		184 - 190	NPIGSEN	0.76	Non-AHT
658.2	658.271	α_{s2}-casein	107 - 111	NPWDQ	0.81	Non-AHT
961.3	961.459	κ-casein	55 - 61	FLPYPYY	1.17	AHT
814.2	814.390		56 - 61	LPYPYY	1	AHT

Table 5. Best ranked cleavage patterns computationally determined of the hydrolysates 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.

Extracellular preparation origin	Cleavage specificity				
	P3	P2	P1	P1'	P2'
L. lactis			F, L or Y	Not P	
			K or R	Not P	
Lb. brevis	Not H, K, R	Not P	Not R	F, L, W, Y	Not P
			F, L or Y	Not P	

Table 6. Angiotensin-converting enzyme-inhibitory activity, expressed as half maximal inhibitory concentration (IC_{50}) from the casein hydrolysates prepared with the extracellular preparations from *Lb. brevis* and *L. lactis*. Activity expressed as values ± mean standard error (n = 3).

Extracellular preparation origin	Casein source	IC_{50} (µg/ml)
L. lactis	Ovine	16 ± 3.1
	Bovine	61 ± 8.6
Lb. brevis	Ovine	40 ± 5.7
	Bovine	70 ± 7.8

Table 7. Sequences identified in the hydrolysates with indication of their reported biological activity and origin in the present work.

Peptide sequence	**Fragment**	**Activity** SBP decrease in SHR/IC_{50}/ opioid receptor selectivity	**Origin**
LHLPLP	β-casein f(133-138)[b,o]	**Antihypertensive**[1] 25.3 mm Hg at 2 mg/kg	*Lb. brevis*
YQKFPQY	α_{s2}-casein f(89-95)[b]; f(90-96)[o]	**Antihypertensive**[2] 15 mm Hg at 2 mg/kg	
RYPSYG	k-casein f(34-39)[b,o]	**Antihypertensive**[3] 17 mm Hg in hydrolysate 300mg/kg	
DAYPSGAW	α_{s1}-casein f(157-164)[b]; f(149-157)[o]	**ACE-inhibitory**[4] IC_{50} 98 µM	
YFYPEL	α_{s1}-CN f(144-149)[b]	**Opioid**[5] µ>δ-receptor	*L. lactis*
LHLPLPL	β-casein f(133-139)[b,o]	**Antihypertensive**[6] 7 mm Hg at 10 mg/kg	Both
YPVEPF	β-casein f(114–119)[b,o]	**Opioid**[7] µ-receptor	
EMPFPK	β-casein f(108 – 113)[b,o]	**ACE-inhibitory**[4] IC_{50} 423 µM	

[b]Bovine protein sequence; [o]Ovine protein sequence

[1] Quirós et al., 2007; [2] Contreras et al., 2009; [3] Jiang et al., 2010; [4] Pihlanto-Leppäla et al 1998; [5] Fernández-Tomé et al., 2016; [6] Miguel et al., 2006 ; [7] Jismaa and Yoshikawa, 1999.
SBP, systolic blood pressure; SHR, spontaneously hypertensive rats ; IC_{50}, half maximal inhibitory concentration; ACE, angiotensin converting enzyme

Figure 1

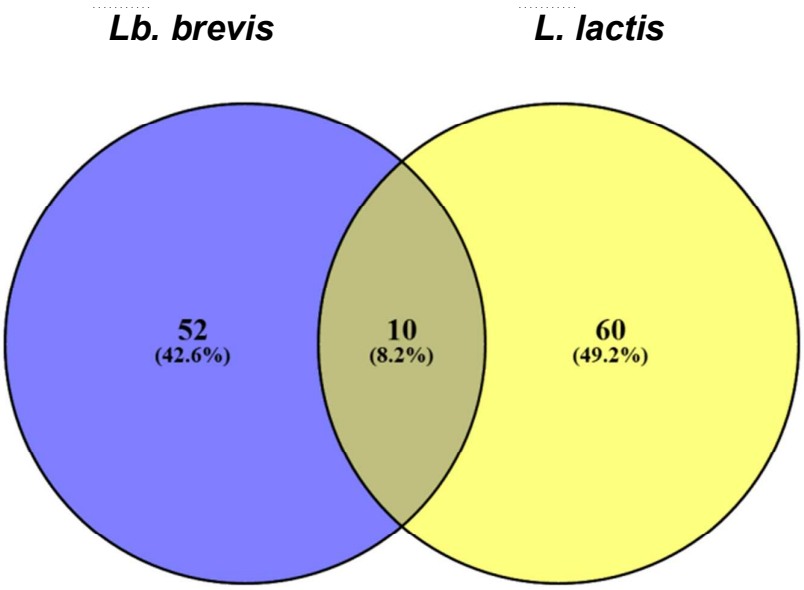
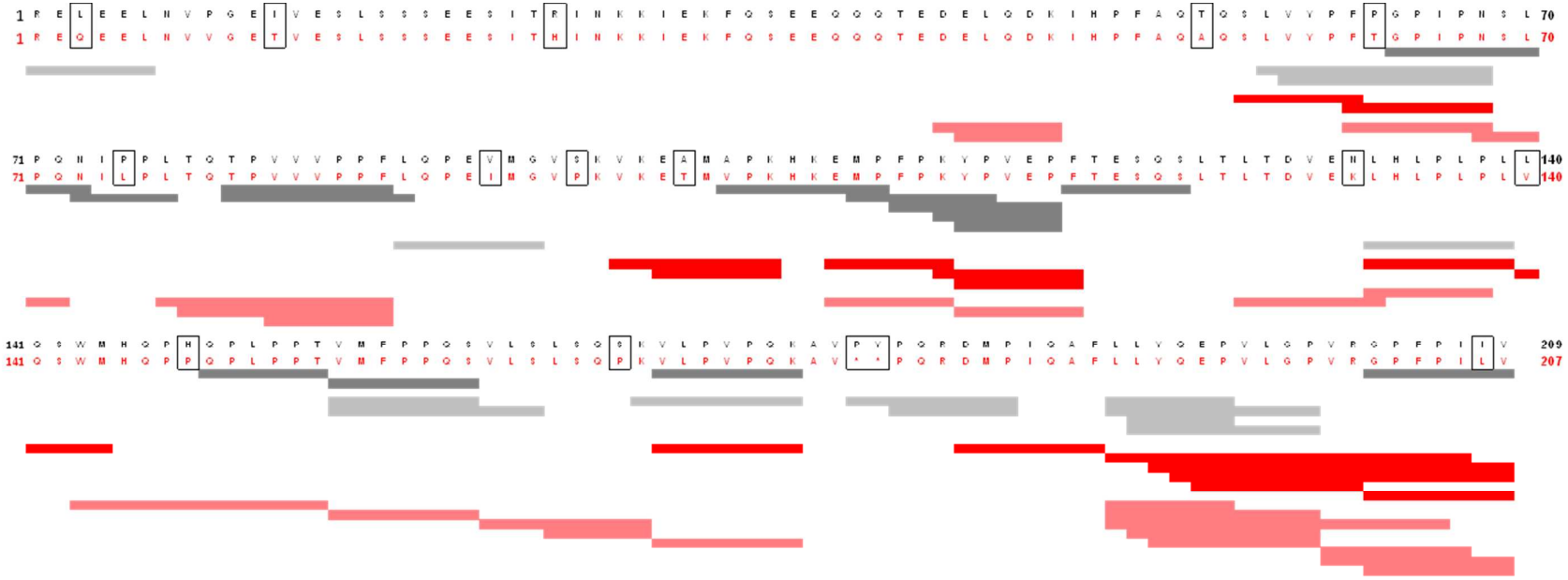
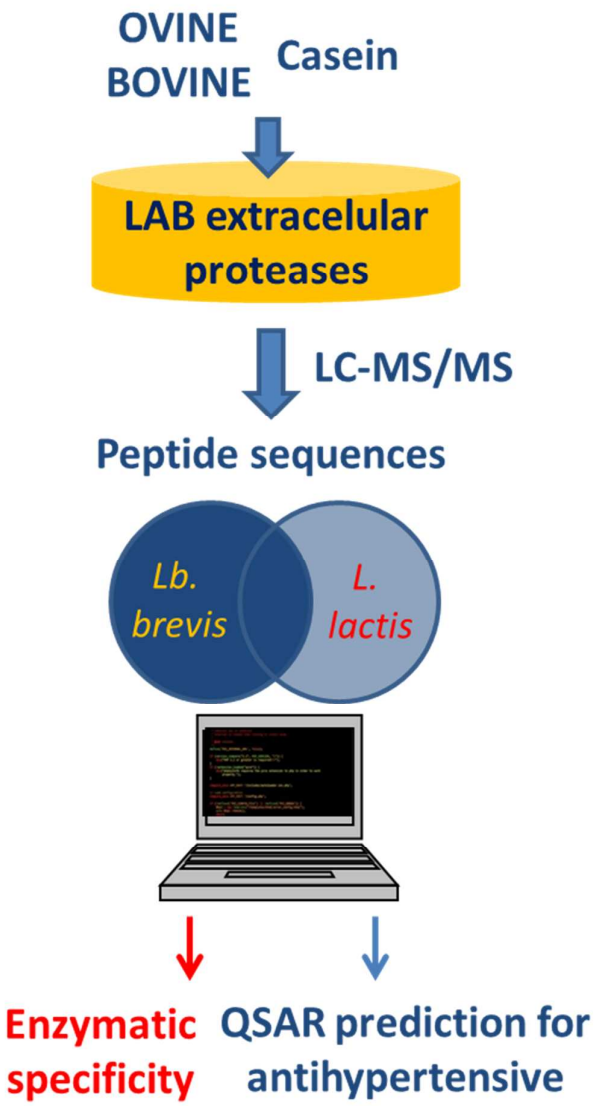


Figure 2



TOC Graphic


OVINE
BOVINE
Casein
LAB extracelular proteases
LC-MS/MS
Peptide sequences
Lb. brevis
L. lactis
Enzymatic specificity
QSAR prediction for antihypertensive