Food Science of Animal Resources

Food Sci. Anim. Resour. 2022 January 42(1):46~60
DOI https://doi.org/10.5851/kosfa.2021.e55

pISSN : 2636-0772 eISSN : 2636-0780
http://www.kosfaj.org

ARTICLE

Angiotensin-I-Converting Enzyme Inhibitory Peptides in Goat Milk Fermented by Lactic Acid Bacteria Isolated from Fermented Food and Breast Milk

Yuliana Tandi Rubak[1,2], Lilis Nuraida[1,3,*], Dyah Iswantini[4,5], and Endang Prangdimurti[1,3]

[1]Department of Food Science and Technology, IPB University (Bogor Agricultural University), IPB Dramaga Campus, Bogor 16680, Indonesia
[2]Agrotechnology Study Program, Faculty of Agriculture, Universitas Nusa Cendana Kupang, UNDANA Lasiana Campus, Kelapa Lima, Nusa Tenggara Timur (NTT) 85228, Indonesia
[3]Southeast Asian Food and Agricultural Science and Technology (SEAFAST) Center, IPB University (Bogor Agricultural University), IPB Dramaga Campus, Bogor 16680, Indonesia
[4]Department of Chemistry, IPB University (Bogor Agricultural University), IPB Dramaga Campus, Bogor 16680, Indonesia
[5]Tropical Biopharmaca Research Center, IPB University (Bogor Agricultural University), IPB Taman Kencana Campus, Bogor 16128, Indonesia

OPEN ACCESS

Received March 16, 2021
Revised September 8, 2021
Accepted September 25, 2021

***Corresponding author** : Lilis Nuraida
Department of Food Science and Technology, IPB University (Bogor Agricultural University), IPB Dramaga Campus, Bogor 16680, Indonesia
Tel: +62-(0251)-8626725
Fax: +62-(0251)-8626725
E-mail: lnuraida@apps.ipb.ac.id

***ORCID**
Yuliana Tandi Rubak
https://orcid.org/0000-0003-4685-1639
Lilis Nuraida
https://orcid.org/0000-0002-0847-5786
Dyah Iswantini
https://orcid.org/0000-0003-1113-2722
Endang Prangdimurti
https://orcid.org/0000-0002-1287-3031

Abstract In this study, angiotensin-I-converting enzyme inhibitory (ACEI) activity was evaluated in fermented goat milk fermented by lactic acid bacteria (LAB) from fermented foods and breast milk. Furthermore, the potential for ACEI peptides was identified in fermented goat milk with the highest ACEI activity. The proteolytic specificity of LAB was also evaluated. The 2% isolate was inoculated into reconstituted goat milk (11%, w/v), then incubated at 37°C until pH 4.6 was reached. The supernatant produced by centrifugation was analyzed for ACEI activity and total peptide. Viable cell counts of LAB and titratable acidity were also evaluated after fermentation. Peptide identification was carried out using nano liquid chromatography mass spectrometry (LC-MS/MS), and potential as an ACEI peptide was carried out based on a literature review. The result revealed that ACEI activity was produced in all samples (20.44%–60.33%). Fermented goat milk of *Lc. lactis* ssp. *lactis* BD17 produced the highest ACEI activity (60.33%; IC_{50} 0.297±0.10 mg/mL) after 48 h incubation, viable cell counts >8 Log CFU/mL, and peptide content of 4.037±0.27/mL. A total of 261 peptides were released, predominantly derived from casein (93%). The proteolytic specificity of *Lc. lactis* ssp. *lactis* BD17 through cleavage on the amino acid tyrosine, leucine, glutamic acid, and proline. A total of 21 peptides were identified as ACEI peptides. This study showed that one of the isolates from fermented food, namely *Lc. lactis* ssp. *lactis* BD17, has the potential as a starter culture for the production of fermented goat milk which has functional properties as a source of antihypertensive peptides.

Keywords angiotensin-I-converting enzyme (ACE) inhibitory activity, antihypertensive peptides, goat milk fermented, proteolytic specificity, *Lc. lactis* ssp. *lactis* BD17

Introduction

Hypertension is a primary risk factor for cardiovascular diseases (CVDs), including stroke, heart attack, heart failure, and other complications related to structural damage to the cardiovascular system. In 2021, the World Health Organization (WHO) reported that CVDs are the leading cause of death globally. People who died from CVDs in 2019 were estimated at 17.9 million, representing 31% of all deaths worldwide. Heart attacks and strokes are the main causes of these deaths (85%) (WHO, 2021). Human blood pressure is regulated by a system called the "renin angiotensin aldosterone system", in which angiotensin-I-converting enzyme inhibition (ACEI) plays an important role. ACE could catalyze the conversion of the decapeptide angiotensin I to the potent vasoconstrictor angiotensin II. Furthermore, this enzyme hydrolyzes bradykinin and stimulates the release of aldosterone which causes vasoconstriction and fluid retention which increases blood pressure (Rai et al., 2017). Therefore, the treatment of clinical hypertension could be done by controlling ACE activity. ACE inhibitors such as captopril, enalapril, alacepril, lisinopril and ramipril are widely used in the clinical treatment of hypertension. However, the use of these synthetic drugs in some cases causes side effects such as coughing, increased blood calcium levels, decreased kidney function, angioedema, and skin rashes (Zeng et al., 2013). Several researchers through *in vivo* studies on rats with spontaneous hypertension (SHR) and humans with hypertension showed that ACE inhibitors without side effects could be obtained from food protein (Bravo et al., 2019; Chen et al., 2014; Seppo et al., 2003).

Food protein from milk and dairy products such as fermented milk is a source of ACEI peptides (Begunova et al., 2021; Wu et al., 2019). Among them, there have been reported from fermented goat milk. The presence of ACE inhibitors in fermented milk is associated with the presence of lactic acid bacteria (LAB). LAB are the dominant group of bacteria involved in fermenting milk such as yogurt and kefir. Kefir is a fermented goat milk that has been consumed for hundreds of years and is believed not only as a source of antihypertensive peptides but also as a source of antioxidants and immunological agents (Ibrahim et al., 2017; Parmar et al., 2020).

During fermentation, milk protein could be hydrolyzed by LAB into peptides and amino acids. The abundance and characteristics of peptides released from milk proteins by LAB are strain-dependent (Wang et al., 2015). Among these peptides, the presence of bioactive peptides could be identified (Li et al., 2017). Bioactive peptides differ in size and sequence. Bioactive peptides that have functional properties as ACEI activity have the characteristics that their molecular weight (MW) is generally <3 kDa and the presence of the amino acids proline and phenylalanine in the sequence (Gonzalez-Gonzalez et al., 2013; Wu et al., 2006).

The ability of LAB to release bioactive peptides in fermented milk (Ayyash et al., 2020; Kim et al., 2017), and the status of LAB as "generally recognized as safe" (GRAS) for application in food, have increased the utilization of certain strains of LAB for production of fermented milk with certain functional properties. The purpose of this study was to investigate ACEI activity in goat milk fermented using LAB from fermented foods and breast milk. The potential of ACEI peptides was identified in the <3 kDa fraction of fermented goat milk with the highest ACEI activity. The proteolytic specificity of the LAB used was also evaluated. The ten strains used were selected because they effectively released ACEI peptides in fermented cow milk in our previous study (Rubak et al., 2020).

Materials and Methods

Lactic acid bacteria

The ten LAB isolates from fermented foods and breast milk used in this study were obtained from culture collections of the

Laboratory of Food Microbiology, Southeast Asian Food and Agricultural Science and Technology (SEAFAST) Center, IPB University (Bogor Agricultural University). The isolates were refreshed in de Man, Rogosa, and Sharpe (MRS) broth and incubated at 37℃ for 24 h, then adapted in fresh skimmed milk for 2 rounds (24 h, 37℃) before being used as a starter culture in the experiment.

Fermentation of goat milk

Goat skimmed milk 11% (w/v) was pasteurized at 95℃ for 10 min. After cooling (45℃), LAB starter culture (2%) was inoculated followed by incubation at 37℃ until pH 4.6 (700 Eutech) was reached. The fermentation process was stopped by heating (75℃ for 1 min) followed by centrifugation (Hettich, Zentrifugen, Mikro 22R) at 6,000×g for 10 min, 4℃. The supernatant was collected for analysis of peptide content and ACEI activity (Cushman and Cheung, 1971). Viable cell counts of LAB and titratable acidity were also analyzed from unheated samples.

Determination of ACEI activity

Hippuryl-L-Histidyl-L-Leucine (HHL, Sigma-Aldrich, St. Louis, MO, USA) was used as an enzyme-substrate. A total of 50 μL of the substrate (50 mM HHL in 0.1 M sodium borate buffer containing 0.3 M NaCl at pH 8.3) was added into a 50 μL sample and incubated at 37℃ for 5 min. To initiate the reaction, 50 μL of 0.1 U/mL ACE (Rabbit lung, Sigma-Aldrich) solution was added, and the mixture was incubated at 37℃ for 5 min. The reaction was stopped by adding 250 μL 1 M HCl. The resulted hippuric acid (HA) was extracted with 1.5 mL ethyl acetate and centrifuged at 2,000×g for 5 min. An aliquot (0.8 mL) of the ethyl acetate layer was transferred to a clean tube and evaporated at 85℃ for 60 min. Distilled water (4 mL) was then added to dissolve the HA in the tube, and the amount of HA formed was measured by measuring the optical density at 228 nm (UV-2800, Hitachi, Tokyo, Japan). The extent of inhibition was calculated as 100% [(B – A) / B] where A is the optical density in the presence of ACE and ACEI components, and B is the optical density without the ACEI component.

IC_{50} and inhibitory efficiency ratio (IER) value

The IC_{50} of the sample having the highest ACEI activity was calculated from the linear regression equation by plotting the ACE inhibition (%) versus the inhibitory concentration for each sample dilution. The percentage of ACEI activity was divided by the peptide concentration to obtain the IER value.

Ultrafiltration

The supernatant of fermented goat milk (4 mL) was pipetted into ultrafiltration centrifuge tubes [molecular weight (MW) cut-off of 3 kDa; Merck, 4 mL, IRL], then centrifuged at 4,000×g for 30 min, 4℃. The fractions (<3 kDa and >3 kDa) were collected and the volume was adjusted to 4 mL by addition of water. Fractions were analyzed for ACEI activity.

Identification of peptides by mass spectrometry

Peptides in <3 kDa fraction were analyzed by using LC Ultimate 3000 series system Tandem Q Exactive Plus Orbitrap HRMS (Thermo Scientific, Dreieich, Germany). The samples (5 μL) were injected into the nano liquid chromatography mass spectrometry (LC-MS/MS) system. The samples were trapped on a trap column (164649, 30 μm×5 mm; Thermo Scientific) and washed for 6 min with a gradient of 98% solvent A [water/acetonitrile (98:2, v/v), 0.1% formic acid] and 2% solvent B

[water/acetonitrile (2:98, v/v), 0.1% formic acid] at a flow rate of 5 µL/min. The eluted peptides were loaded and separated on a capillary column (PepMap RSLC-C18, 75-µm×150 mm, 3.5 µm particle size, 100 pore size, Thermo Scientific, ES800) at a flow rate of 300 nL/min with a gradient at 2% to 35% solvent B over 30 min, then from 35% to 90% over ten min, followed by 90% solvent B for 5 min, and finally 5% solvent B for 15 min. Electrospray was performed at an ion spray voltage of 3500 eV. Automatically, the peptides were analyzed using Proteomic Discoverer 2.2 software. The range of m/z values was 200–2,000.

Identification of ACEI peptides was carried out through a literature search. The target of investigation is a peptide that provides 100% similarity to the ACEI peptide that has been previously reported by the researchers.

Statistical analysis

The data were analyzed using Analysis of Variance (ANOVA) performed using SPSS version 22. $p \leq 0.05$ was considered significant. Each experiments were repeated three times, and the data were presented as mean±SD.

Result and Discussion

Characteristics of goat milk fermented by lactic acid bacteria from fermented food and breast milk

Milk has been known as a suitable growth medium for LAB. The population of the ten LAB in fermented goat milk reached 9 Log CFU/mL. Viable cell counts of the LAB ranged from 9.18±0.46 to 9.79±0.39 Log CFU/mL. When the pH reached 4.6, there was no difference in population (Table 1), which is in accordance with previous results obtained by Elkhtab et al. (2017) and from fermented cow milk using similar cultures (Rubak et al., 2020). However, the fermentation time to reach pH 4.6 was different between isolates that ranged from 18 to 48 h with titratable acidity ranging from 0.77±0.06 to 0.94±0.04%. A short fermentation time (18 h) was observed in the fermentation by *Lactobacillus rhamnosus* R2, while the longest fermentation time (48 h) occurred in the fermentation by *Lactobacillus fermentum* S206, *Lactobacillus delbrueckii* BD7, and *Lactococcus lactis* ssp. *lactis* BD17. In our research the fermentation was ended at pH 4.6 to obtain high production of peptides. The release of a bioactive peptide from the protein matrix by culture could decrease when the pH value falls below 4.5 (Gonzalez-Gonzalez et al., 2013). Increase in coagulation could inhibit bacterial cell diffusion to protein tissue,

Table 1. Profile of goat milk fermented by LAB from kefir grains and breast milk

Culture	Fermentation time to reach pH 4.6 (h)	Titratable acidity (%)	Viable cell count (Log CFU/mL)
Lactobacillus rhamnosus R2	18	0.90±0.01	9.69±0.11
Pediococcus pentasaceus 1 W2SR04	24	0.94±0.04	9.18±0.46
Lactobacillus kefiri YK4	24	0.80±0.03	9.79±0.39
Lactobacillus kefiri JK17	24	0.79±0.02	9.67±0.07
Lactobacillus fermentum R6	24	0.84±0.01	9.58±0.03
Lactobacillus plantarum 1W22408	32	0.77±0.06	9.25±0.39
Lactobacillus R7F	36	0.81±0.02	9.46±0.52
Lactobacillus fermentum S206	48	0.70±0.06	9.53±0.17
Lactobacillus delbrueckii BD7	48	0.75±0.05	9.73±0.36
Lactococcus lactis ssp. *lactis* BD17	48	0.75±0.05	9.55±0.38

LAB, lactic acid bacteria.

thus inhibiting access of *Cell Envelope Proteinase* (CEP) to milk protein for hydrolysis. Further acidification could be avoided by stopping fermentation when it reaches pH 4.6, or the pH must be controlled by adding alkaline solutions such as sodium hydroxide (Chen et al., 2015).

Angiotensin-I-converting enzyme inhibitory (ACEI) activity

ACEI activity was detected in all supernatants of goat milk fermented in the range of 20.44±2.33 to 60.79±8.78% (Table 2). The highest percentage of ACEI activity (>50%) was obtained in goat milk fermented by *Lb. delbrueckii* BD7 and *Lc. lactis* ssp. *lactis* BD17, but it was not significantly different (>0.05) from that of *Lb. kefiri* YK4 and *Lb. kefiri* JK17. It has been reported that goat milk could be used as a potential precursor for the production of ACE inhibitors through the fermentation process (Izquierdo-González et al., 2019). Starter cultures of LAB, growth conditions, and substrate are factors that influence ACEI production in fermented milk (Li et al., 2017; Shu et al., 2015; Wang et al., 2015). *Lactobacillus* species are known to produce high ACEI activity (>50%) in fermented milk (Hati et al., 2018; Wu et al., 2019). The variation of ACEI activity between LAB in milk fermented is related to its proteolytic activity. The proteolytic activity of LAB is determined by the specificity of its proteolytic components (Chen et al., 2015).

The proteolytic activity of LAB in goat milk was measured by the OPA method, with results ranging from 3.55±0.26 to 5.69±0.21 mg/mL (Table 2). Goat milk fermented by *P. pentasaceus* 1 W2SR04 and *Lb. kefiri* YK4 (>5 mg/mL) showed high peptide content. In a study by Toe et al. (2019), *P. pentasaceus* species also showed high proteolytic activity. However, high peptide content was not always associated with high ACEI activity in samples. This is also seen in the results of our study. ACEI activity is more related to the abundance of ACEI peptides that could be released during fermentation.

The IER values evaluated in ten samples showed that the highest IER values were obtained in fermented goat milk of *Lc. lactis* ssp. *lactis* BD17. The IC_{50} value was also determined in this sample and the result was 0.297±0.10 mg/mL. The IC_{50} value reflects the peptide concentration required to inhibit 50% ACE. The IC_{50} value in fermented milk <1 mg/mL (Gútiez et al., 2013). Our results show that the obtained IC_{50} value is lower than that of fermented milk of other *Lactobacillus* species, as reported by Qian et al. (2011) in fermented milk by *Lb. delbrueckii* (IC_{50} 67.71±7.62 mg/mL), by Moslehishad et al. (2013) in fermented milk by *Lb. rhamnosus* (IC_{50}: 3.947±0.029 mg/mL) and by Chen et al. (2007) in fermented milk using

Table 2. ACE inhibitory activity, peptide content, and IER of goat milk fermented by LAB after reaching pH 4.6

Culture	Inhibition of ACE (%)	Peptide content (mg/mL)	IER (% per mg/mL)
Pediococcus pentasaceus 1 W2SR04	20.44±2.33^{c}	5.696±0.21^{a}	3.60±0.49^{c}
Lactobacillus kefiri YK4	58.65±8.87^{a}	5.691±0.25^{a}	10.31±1.55^{b}
Lactobacillus fermentum S206	26.64±3.90^{c}	4.809±0.23^{b}	5.59±1.04^{c}
Lactobacillus delbrueckii BD7	60.79±8.78^{a}	4.767±0.27^{b}	12.81±2.12^{ab}
Lactobacillus kefiri JK17	56.94±2.81^{a}	4.750±0.30^{b}	12.06±1.23^{b}
Lactobacillus fermentum R6	48.50±6.92^{ab}	4.089±0.10^{c}	11.83±1.42^{b}
Lactobacillus R7F	42.85±5.10^{b}	4.007±0.23^{c}	10.66±0.73^{b}
Lactococcus lactis ssp. *lactis* BD17	60.33±4.73^{a}	4.037±0.27^{c}	14.99±1.26 ^{a}
Lactobacillus rhamnosus R2	40.51±6.15^{b}	3.658±0.19^{c}	11.03±1.10^{b}
Lactobacillus plantarum I W22408	39.69±4.70^{b}	3.548±0.26^{c}	11.15±0.53^{b}

$^{a–c}$ Different superscript in the same column indicated significant ($p<0.05$).
ACE, angiotensin-I-converting enzyme; IER, inhibitory efficiency ratio; LAB, lactic acid bacteria.

several isolates (IC_{50}: 0.65 mg/mL) and in koumiss (52.47±2.87 mg/mL) (Chen et al., 2010). Barla et al. (2016) have also reported from fermented milk by *Lb. brevis*, *Lb. buchnery* and *W. hellenica* (IC_{50}: 0.28–0.83 mg/mL).

ACEI activity of >3 kDa and <3 kDa fraction

Filtration using a 3 kDa MW cut-off showed that the ACEI activity was concentrated in the MW fraction <3 kDa (Table 3). There was no significant difference (>0.05) in the ACEI activity of the <3 kDa fraction compared to the supernatant (without filtration). Bioactive peptides with ACEI activity have been reported as peptides with MW of <3 kDa (Gonzalez-Gonzalez et al., 2013).

Characteristics of peptides in the <3 kDa fraction of fermented goat milk of *Lc. lactis* ssp. *lactis* BD17

A total of 261 peptides were released in fermented goat milk by *Lc. lactis* ssp. *lactis* BD17 (Table 4 and Supplementary Data). Most of the peptides were hydrolyzed from casein (97%) and whey (3%). The main fraction of goat milk protein is casein, which is 80% of the total milk protein (Jandal, 1996). This explains the abundance of peptide hydrolyzed from casein in our results. Another thing that casein has a very flexible and open structure so it is very sensitive to proteolysis. While whey protein is more resistant which is explained by the presence of a globular structure (Swaisgood, 1993). According to the results, β-casein (54.02%) was the most accessible to the proteolytic system of *Lc. lactis* ssp. *lactis* BD17 to release a number of peptides. The cleavage site's dominance on the β-casein was also shown by *Lb. rhamnosus* CGMCC11055 (Guo et al., 2016) and *L. delbrueckii* subsp. *lactis* ACA-DC 178 (Hebert et al., 2008).

Table 3. ACE inhibitory activity of supernatant (without ultrafiltration), fractions of >3 kDa and <3 kDa of goat milk fermented by *Lc. lactis* ssp. *lactis* BD17

ACE inhibitory activity (%)		
Supernatant (Without ultrafiltration)	>3 kDa	<3 kDa
60.33±4.73[a]	24.57±2.36[b]	57.31±2.41[a]

ACE, angiotensin-I-converting enzyme.

Table 4. Characteristics of peptides (<3 kDa) of fermented goat milk of *Lc. lactis* ssp. *lactis* BD17

Parent protein*	Total peptides	Range of precursor (m/z)	Range of precussor (MW)	Amino acid residue	Dominant peptide
α_{S1}-Casein	41	330.1–1,146.0	659.3–2,291.1	6–20	FSDIPNPIGSE, FSDIPNPIGSENSGKTTMP, NSGKTTMPLW
α_{S2}-Casein	37	310.1–906.9	772.4–1,812.9	6–15	QGPIVLNPWDQVKR
β-Casein	141	326.7–1,072.5	652.40–2,201.1	6–18	QEPVLGPVRGPFPII, QEPVLGPVRGPFPI, QEPVLGPVRGPFPIIV, VLGPVRGPFPIIV, TQTPVVVPPFLQPE
κ-Casein	36	376.5–750.8	761.45–1,500.7	6–10	ARHPHPHLSFM
ά-Lactalbumin	3	497.7–563.7	994.4–1,126.4	8–10	AFHTSGYDTQ
β-Lactoglobulin	3	420.5–482.2	903.57–1,259.7	8–11	

* Protein access code at https://www.uniprot.org/.
A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glysin; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonin; V, valine; W, triptophan; Y, tyrosine; MW, molecular weight.

The spectrum of MS analysis revealed a large number of peaks with retention times (RTs) of 8 to 65 min (Fig. 1), representing an abundance of released peptides with peptide mass/molecular charge (m/z) ranging from 310.1 to 1,146.0. The diversity of detected ions indicates that the nine peaks with RT of 11.29 to 30.97 corresponded to 13 peptides identified in goat milk fermented by *Lc. lactis* ssp. *lactis* BD17 (Table 5). ARHPHPHLSFM (κ-casein; RT 11.29, 11.89; m/z 665.33, 673.33) was a peptide that exhibited a prominent peak according to its abundance in the sample. This peptide was also identified as being present in goat milk kefir (Izquierdo-González et al., 2019) and goat milk (Ibrahim et al., 2017). Another signal was associated with the abundance of peptides in goat milk fermented by *Lc. lactis* ssp. *lactis* BD17 from the parent protein β-casein, namely MPFPKYPVEPF (RT 20.54; m/z 676.34), QEPVLGPVRGPFPI (RT 29.61; m/z 753.43) and RDMPIQAFLL (RT 23.59; m/z 602.33). This peptide has also been identified in goat milk kefir (Izquierdo-González et al., 2019) and bovine kefir (Ebner et al., 2015).

The release of peptides from the protein matrix is initiated by CEP, one of the essential enzymes in the LAB proteolytic system (Griffiths and Tellez, 2013) which cleaves the proteins resulting in peptides with 4 to 30 amino acids. The CEP of LAB is classified into three types based on the hydrolysis of casein (Kunji et al., 1996): (1) CEP type PI which specifically hydrolyzes β-casein, (2) CEP type PIII which hydrolyzes αS1-casein and κ-casein, (3) CEP intermediate type PI/PIII which hydrolyzes β-casein and αS1-casein. Based on this classification, our results indicate that the CEP of *Lc. lactis* ssp. *lactis* BD17 represents CEP types I and III. These types of CEP were also reported from *Lb. casei* PRA205 and *Lb. rhamnosus* PRA331 (Solieri et al., 2018), and *Lb. paracasei* ssp. *paracasei* (Nikolić et al., 2009).

Types and domains in the CEP region of each LAB provide variations in the specificity of the hydrolyzed substrate which have implications for the diversity of MWs and amino acid sequences of the released peptides (Raveschot et al., 2020). The MW of the peptide released by *Lc. lactis* ssp. *lactis* BD17 ranged from 659 to 2,201.1 dalton with amino acid residues

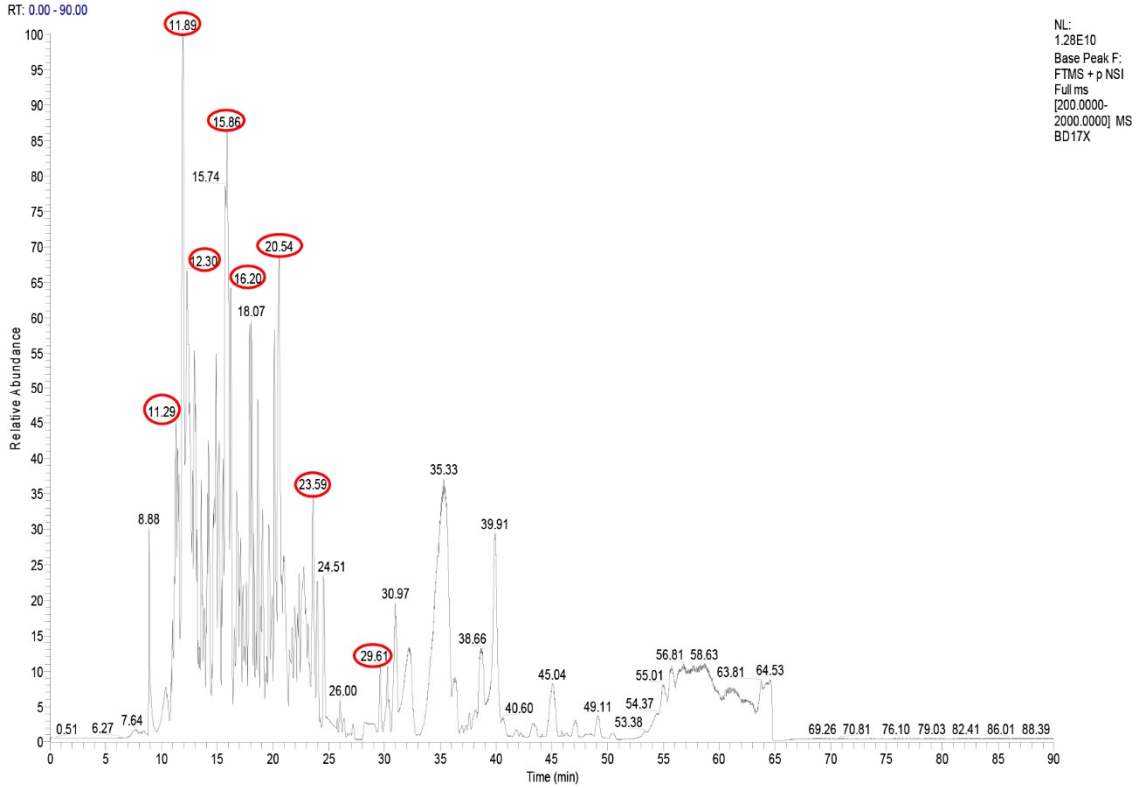

Fig. 1. Nano liquid chromatography mass spectrometry (LC-MS/MS) chromatogram of goat milk fermented by *Lc. lactis* ssp. *lactis* BD17.

Table 5. Peptides identified of goat milk fermented by *Lc. lactis* ssp. *lactis* BD17 with retention time (RT) detected on nano liquid chromatography mass spectrometry (LC-MS/MS). Peptides in bold letters indicate a match with RT

Retention time (min)	Retention time (min)	m/z [Da]	MH^+ [Da]	Peptide identified	Parent protein
11.29	11.29	665.33	1,329.66	**ARHPHPHLSFM**	κ-Casein (A0A140T8A9*)
	11.22	495.75	990.49	ITVDDKHY	α_{s2}-Casein (P02663*)
11.89	11.81	563.74	1,126.48	AFHTSGYDTQ	ά-Lactalbumin (B6V3I5*)
	11.87	581.34	1,161.67	EKNRLNFLK	α_{s2}-Casein (P02663*)
	11.89	673.33	1,345.66	**ARHPHPHLSFM**	κ-Casein (A0A140T8A9*)
12.30	12.30	559.75	1,118.49	**QQQTEDELQ**	β-Casein (P02666*)
	12.30	546.79	1,092.57	**YQKALNEIN**	α_{s2}-Casein (P02663*)
15.86	15.86	470.74	940.47	**IPNPIGSEN**	α_{s1}-Casein (P02662*)
	15.86	521.27	1,041.54	**RYLGYLEQ**	α_{s1}-Casein (P02662*)
16.20	16.20	413.72	826.43	**IPNPIGSE**	α_{s1}-Casein (P02662*)
	16.20	560.78	1,120.55	**FYQKFPQY**	α_{s2}-Casein (P02663*)
	16.24	621.31	1,241.61	DELQDKIHPF	β-Casein (P02666*)
	16.26	457.24	913.48	RYLGYLE	α_{s1}-Casein (P02662*)
	16.26	610.29	1,219.57	SRYPSYGLNY	κ-Casein (A0A140T8A9*)
20.54	20.54	676.34	1,351.68	**MPFPKYPVEPF**	β-Casein (P02666*)
	20.59	329.20	657.40	VVVPPF	β-Casein (P02666*)
23.59	23.50	683.86	1,366.72	RDMPIQAFLLY	β-Casein (P02666*)
	23.51	559.80	1,118.59	MFPPQSVLSL	β-Casein (P02666*)
	23.53	385.74	770.48	VVVPPFL	β-Casein (P02666*)
	23.54	948.04	1,895.07	LYQEPVLGPVRGPFPII	β-Casein (P02666*)
	23.56	795.47	1,589.94	EPVLGPVRGPFPIIV	β-Casein (P02666*)
	23.59	602.33	1,203.66	**RDMPIQAFLL**	β-Casein (P02666*)
24.51	24.50	859.50	1,718.00	QEPVLGPVRGPFPIIV	β-Casein (P02666*)
	24.55	484.79	968.58	TPVVVPPFL	β-Casein (P02666*)
26.00	26.07	548.30	1,095.59	MPIQAFLLY	β-Casein (P02666*)
	26.08	809.97	1,618.93	QEPVLGPVRGPFPII	β-Casein (P02666*)
29.61	29.61	753.42	1,505.84	**QEPVLGPVRGPFPI**	β-Casein (P02666*)
30.97	30.98	776.42	1,551.83	TQTPVVVPPFLQPE	β-Casein (P02666*)

* Protein access code at https://www.uniprot.org/.
A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glysin; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; r, arginine; s, serine; t, threonin; v, valine; W, triptophan; Y, tyrosine.

ranging from 6 to 20. An investigation was carried out to determine the specificity of cleavage of CEP *Lc. lactis* ssp. *lactis* BD17 in goat milk parent protein during fermentation to release a number of peptides. The results are presented in Fig. 2. It appears that sites of *Lc. lactis* ssp. *lactis* BD17 dispersed throughout the parent protein, although certain amino acids are favorite for cleavage by CEP of *Lc. lactis* ssp. *lactis* BD17. In the αS1-casein region, the cleavage sites were frequently at serine, aspartate, and phenylalanine amino acid (f183-f184, f186-f187, and f184-f185), in αS2-casein region the sites were at

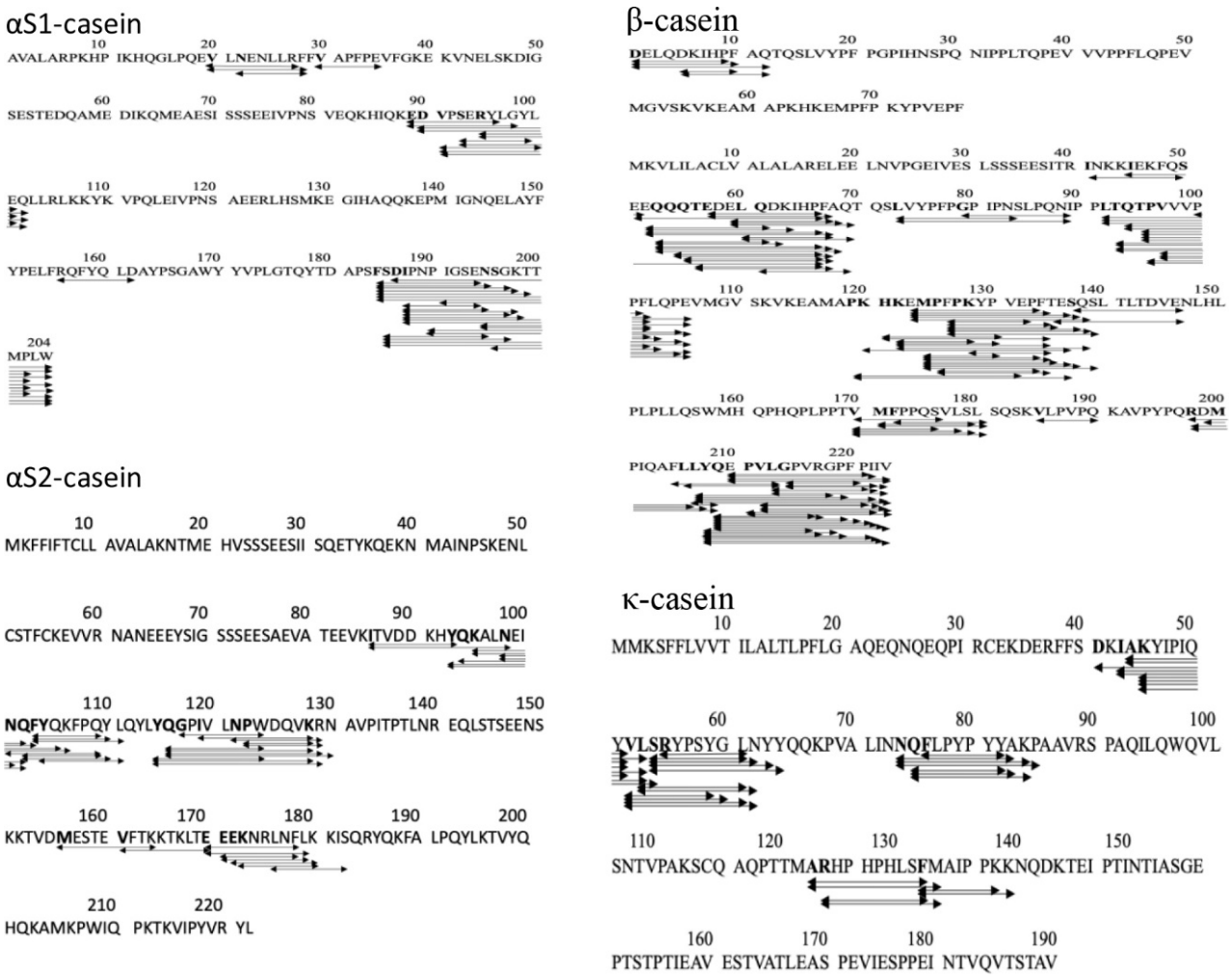

Fig. 2. Cleavage specifics of the peptide of the parent protein α_{s1}-casein, α_{s2}-casein, β-casein, and κ-casein.

amino acids tyrosine, leucine, and glutamine (f116-f117, f114-f115, f112-f113), and in the β-casein region the sites were at amino acids tyrosine, leucine, glutamic acid and proline (f208-f209, f207-f208, f123-f124, f125-f126). Moreover, in a κ-casein region the cleavage sites were at amino acids leucine, tyrosine, and alanine (f53-f54, f51-f52, f44-f45). These results indicate that the cleavage sites of casein by *Lc. lactis* ssp. *lactis* BD17 occur mostly in hydrophobic and aromatic amino acids. It seems that hydrophobic and aromatic amino acids are more easily accessed and released from parent protein. Similar results have been reported by Lozo et al. (2011) on *Lb. paracasei* subsp. *paracasei* BGHN14 (prtp), *Lb. rhamnosus* BGT10 (prtR), and *Lb. helveticus* BGRA43 (prtP), and Hebert et al. (2008) reported on *Lb. delbrueckii* subsp. *lactis* CRL 581.

ACEI peptides

Twenty-one of the 261 peptides released by *Lc. lactis* ssp. *lactis* BD17 were identified as ACEI peptides (Table 6), most of which were released from β-casein. One of peptides namely ARHPHPHLSFM from parent protein κ-casein; 116-f117 was reported as an ACEI peptide (Ibrahim et al., 2017), and VLNENLR (α_{S1}-casein; f39-f40) (Swaisgood, 1993). ACEI peptides could be identified based on their amino acid sequence (Lunow et al., 2015).

The three amino acids located at the C-terminus could determine whether a peptide could act as an ACEI peptide (Wu et al., 2006). Amino acids from aliphatic groups (proline, isoleucine, valine) and aromatic amino acids (phenylalanine) are the

Table 6. ACE inhibitory peptides in fermented goat milk of *Lc. lactis* ssp. *lactis* BD17 as reported in the literature

Parent protein*	Sequence	m/z [Da]	MH+ [Da]	Charge	References
κ-Casein	ARHPHPHLSFM	665.33	1,329.66	3	Ibrahim et al. (2017)
β-Casein	DELQDKIHPF	621.30	1,241.61	2	Rodríguez-Figueroa et al. (2012)
β-Casein	DKIHPF	378.71	756.41	2	Fan et al. (2019)
β-Casein	DKIHPFAQ	478.25	955.50	2	Gobbetti et al. (2000)
β-Casein	EMPFPKYPVEPF	740.86	1,480.71	2	Papadimitriou et al. (2007)
β-Casein	ELQDKIHPF	563.80	1,126.59	2	Fan et al. (2019)
β-Casein	GPVRGPFPI	470.27	939.54	2	Amorim et al. (2019)
β-Casein	LGPVRGPFP	470.27	939.54	2	Hernández-Ledesma et al. (2004)
β-Casein	LTQTPVVVPPF	599.34	1,197.68	2	Villegas et al. (2014)
β-Casein	LVYPFPGPIHNSLPQN	896.96	1,792.93	2	Quirós et al. (2009)
β-Casein	LYQEPVLGPVRGPFPIIV	997.58	1,994.14	2	Pihlanto et al. (2010)
β-Casein	MPFPKYPVEP	602.80	1,204.60	2	Contreras et al. (2009)
β-Casein	MPFPKYPVEPF	676.34	1,351.67	2	Hayes et al. (2007)
β-Casein	QEPVLGPVRGPFP	696.88	1,392.76	2	Hernández-Ledesma et al. (2004)
β-Casein .	QEPVLGPVRGPFPIIV	859.50	1,718.00	2	Perpetuo et al. (2003)
α_{s1}-Casein	VLNENLR	485.78	970.56	2	Zhao et al. (2019)
β-Casein	VLGPVRGPFP	519.81	1,038.61	2	Gútiez et al. (2013)
β-Casein	VVVPPF	329.20	657.39	2	Torres-Llanez et al. (2011)
β-Casein	YQEPVLGPVRGPFPI	834.95	1,668.90	2	Zhao et al. (2019)
β-Casein	YQEPVLGPVRGPFPIIV	627.69	1,881.06	3	Zhao et al. (2019)
β-Casein	YQEPVLGPVR	579.31	1,157.63	2	Kalyankar et al. (2013)

* Protein access code at https://www.uniprot.org/.
ACE, angiotensin-I-converting enzyme; A, alanine; D, aspartic acid; E, glutamic acid; F, phenylalanine; G, glysin; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonin; V, valine; W, triptophan; Y, tyrosine; cas, casein.

dominant amino acids found in the ACEI peptide. Our investigation of other peptide released by *Lc. lactis* ssp. *lactis* BD17 which has not been identified as an ACEI peptide according to a literature search, demonstrated its potential as an ACEI peptide. A total of 36% of these peptides had a proline amino acid residue and 21% a phenylalanine amino acid residue at the C-terminus.

The characteristics of the ACEI peptide were not only observed in the presence of amino acids at the C-terminus. By other researchers, the presence of amino acids at the N-terminal was also evaluated. Aslam et al. (2019) showed that three identified ACEI peptides were released in goat milk fermented by *Lb. helveticus* cicc22171 has hydrophobic/aliphatic amino acids not only at the C-terminus but also at the N-terminus (valine and proline). Daliri et al. (2018) also presented their research results that four peptides identified as ACEI peptides were associated with the presence of negative amino acids (glutamate) and uncharged amino acids (glutamine) at their N-terminus. The presence of this amino acid in the *Lc. lactis* ssp. *lactis* BD17 peptide was also identified, namely in the peptide released from the parent protein β-casein and α_{S1}-casein (Table 4).

In addition to the presence of certain amino acids in the ACEI peptide sequence, another characteristic of ACEI peptides is their MW. ACEI peptides are generally short peptides with MW <3 kDa, may consist of 6 to 16 amino acids (Ibrahim et al., 2017). However, ACEI peptides with 20 amino acid residues have also been reported (Elkhtab et al., 2017). The ACEI peptide identified in our study has a MW of <2 kDa (657–1,994 Da), consisting of 6 to 18 amino acids. Short peptides are known to easily bind to the active site of ACE (Aslam et al., 2019). ACEI peptide binding to the active of ACE is facilitated by hydrogen bonding, hydrophobic interactions, and disrupting the stability of the Zn^{2+} ion. The presence of ACEI peptide in fermented milk is highly dependent on the type of LAB used for the fermentation process. It is therefore very important to use isolates that have been shown to have the ability to release ACEI peptides. *Lc. lactis* ssp. *lactis* BD17 used in this study was an isolate isolated from kefir. Although the ability of this strain has only been explored in this study, as a comparison, the results of studies using kefir grain could be presented. Ebner et al. (2015) stated that kefir microbes were able to release 12 ACEI peptides in fermented milk. The same thing was also conveyed by Dallas et al. (2016) that kefir microbes release 29 bioactive peptides, including ACEI peptides in fermented cow milk. A recent study by Izquierdo-González et al. (2019) showed that in goat milk using kefir grains, five ACEI peptides were identified.

Conclusion

ACEI activity was detected in all fermented goat milk using isolates from fermented food and breast milk. Goat milk fermented using *Lc. lactis* ssp. *lactis* BD17 produced the highest ACEI activity (IC_{50}: 0.297±0.10 mg/mL) after 48 h of incubation. A total of 261 peptides were hydrolyzed by *Lc. lactis* ssp. *lactis* BD17 during fermentation, most of which were released from casein (β-casein). The peptide has a MW of 659 to 2,201.1 dalton, consisting of 6–20 amino acid residues. The CEP specificity of *Lc. lactis* ssp. *lactis* BD17 towards goat milk parent protein, was dominated by cleavage on the amino acids tyrosine, leucine, glutamic acid, and proline. A total of 21 peptides were identified as ACEI peptides, having 100% homology to the reported ACEI peptides. Several characteristics of ACEI peptides are present in peptides hydrolyzed by *Lc. lactis* ssp. *lactis* BD17. These peptides mostly have hydrophobic and aromatic amino acids at the C-terminus. The results of this study add to the information that *Lc. lactis* ssp. *lactis* BD17 is a candidate that could be considered as a starter culture to obtain fermented milk that has functional properties as a source of ACEI peptides.

Supplementary Materials

Supplementary materials are only available online from: https://doi.org/10.5851/kosfa.2021.e55.

Conflicts of Interest

The authors declare no potential conflicts of interest.

Acknowledgements

The author would like to thank the LPDP (Lembaga Pengelola Dana Pendidikan), Ministry of Finance, Republic of Indonesia for providing research funding under the scheme of Scholarship of Beasiswa Unggulan Dosen Indonesia, Dalam Negeri.

Author Contributions

Conceptualization: Rubak YT, Nuraida L, Iswantini D, Prangdimurti E. Data curation: Rubak YT, Nuraida L, Iswantini D, Prangdimurti E. Formal analysis: Rubak YT, Nuraida L. Methodology: Rubak YT, Nuraida L, Iswantini D, Prangdimurti E. Software: Rubak YT, Nuraida L. Validation: Rubak YT, Nuraida L, Iswantini D, Prangdimurti E. Investigation: Rubak YT, Nuraida L, Iswantini D, Prangdimurti E. Writing - original draft: Rubak YT, Nuraida L. Writing - review & editing: Rubak YT, Nuraida L, Iswantini D, Prangdimurti E.

Ethics Approval

This article does not require IRB/IACUC approval because there are no human and animal participants.

References

Amorim M, Marques C, Pereira JO, Guardão L, Martins MJ, Osório H, Moura D, Calhau C, Pinheiro H, Pintado M. 2019. Antihypertensive effect of spent brewer yeast peptide. Process Biochem 76:213-218.

Aslam MZ, Shoukat S, Hongfei Z, Bolin Z. 2019. Proteomic analysis of ACE inhibitory peptides extracted from fermented goat milk. Int J Pept Res Ther 25:1259-1270.

Ayyash M, Olaimat A, Al-Nabulsi A, Liu SQ. 2020. Bioactive properties of novel probiotic *Lactococcus lactis* fermented camel sausages: Cytotoxicity, angiotensin converting enzyme inhibition, antioxidant capacity, and antidiabetic activity. Food Sci Anim Resour 40:155-171.

Barla F, Koyanagi T, Tokuda N, Matsui H, Katayama T, Kumagai H, Michihata T, Sasaki T, Tsuji A, Enomoto T. 2016. The γ-aminobutyric acid-producing ability under low pH conditions of lactic acid bacteria isolated from traditional fermented foods of Ishikawa Prefecture, Japan, with a strong ability to produce ACE-inhibitory peptides. Biotechnol Rep 10:105-110.

Begunova AV, Savinova OS, Glazunova OA, Moiseenko KV, Rozhkova IV, Fedorova TV. 2021. Development of antioxidant and antihypertensive properties during growth of *Lactobacillus helveticus*, *Lactobacillus rhamnosus* and *Lactobacillus reuteri* on cow's milk: Fermentation and peptidomics study. Foods 10:17.

Bravo FI, Mas-Capdevila A, Margalef M, Arola-Arnal A, Muguerza B. 2019. Novel antihypertensive peptides derived from chicken foot proteins. Mol Nutr Food Res 63:1801176.

Chen GW, Tsai JS, Sun Pan B. 2007. Purification of angiotensin I-converting enzyme inhibitory peptides and antihypertensive effect of milk produced by protease-facilitated lactic fermentation. Int Dairy J 17:641-647.

Chen Y, Li C, Xue J, Kwok L, Yang J, Zhang H, Menghe B. 2015. Characterization of angiotensin-converting enzyme inhibitory activity of fermented milk produced by *Lactobacillus helveticus*. J Dairy Sci 98:5113-5124.

Chen Y, Liu W, Xue J, Yang J, Chen X, Shao Y, Kwok L, Bilige M, Mang L, Zhang H. 2014. Angiotensin-converting enzyme inhibitory activity of *Lactobacillus helveticus* strains from traditional fermented dairy foods and antihypertensive effect of fermented milk of strain H9. J Dairy Sci 97:6680-6692.

Chen Y, Wang Z, Chen X, Liu Y, Zhang H, Sun T. 2010. Identification of angiotensin I-converting enzyme inhibitory peptides from koumiss, a traditional fermented mare's milk. J Dairy Sci 93:884-892.

Contreras MM. Carrón R, Montero MJ, Ramos M, Recio I. 2009. Novel casein-derived peptides with antihypertensive

activity. Int Dairy J 19:566-573.

Cushman DW, Cheung HS. 1971. Spectrophotometric assay and properties of the angiotensin-converting enzyme of rabbit lung. Biochem Pharmacol 20:1637-1648.

Daliri EBM, Lee BH, Park MH, Kim JH, Oh DH. 2018. Novel angiotensin I-converting enzyme inhibitory peptides from soybean protein isolates fermented by *Pediococcus pentosaceus* SDL1409. LWT-Food Sci Technol 93:88-93.

Dallas DC, Citerne F, Tian T, Silva VLM, Kalanetra KM, Frese SA, Robinson RC, Mills DA, Barile D. 2016. Peptidomic analysis reveals proteolytic activity of kefir microorganisms on bovine milk proteins. Food Chem 197:273-284.

Ebner J, Aşçı Arslan A, Fedorova M, Hoffmann R, Küçükçetin A, Pischetsrieder M. 2015. Peptide profiling of bovine kefir reveals 236 unique peptides released from caseins during its production by starter culture or kefir grains. J Proteomics 117:41-57.

Elkhtab E, El-Alfy M, Shenana M, Mohamed A, Yousef AE. 2017. New potentially antihypertensive peptides liberated in milk during fermentation with selected lactic acid bacteria and kombucha cultures. J Dairy Sci 100:9508-9520.

Fan H, Liao W, Wu J. 2019. Molecular interactions, bioavailability, and cellular mechanisms of angiotensin-converting enzyme inhibitory peptides. J Food Biochem 43:e12572.

Gobbetti M, Ferranti P, Smacchi E, Goffredi F, Addeo F. 2000. Production of angiotensin-I-converting-enzyme-inhibitory peptides in fermented milks started by *Lactobacillus delbrueckii* subsp. *bulgaricus* SS1 and *Lactococcus lactis* subsp. *cremoris* FT4. Appl Environ Microbiol 66:3898-3904.

Gonzalez-Gonzalez C, Gibson T, Jauregi P. 2013. Novel probiotic-fermented milk with angiotensin I-converting enzyme inhibitory peptides produced by *Bifidobacterium bifidum* MF 20/5. Int J Food Microbiol 167:131-137.

Griffiths MW, Tellez AM. 2013. *Lactobacillus helveticus*: The proteolytic system. Front Microbiol 4:30.

Guo T, Ouyang X, Xin Y, Wang Y, Zhang S, Kong J. 2016. Characterization of a new cell envelope proteinase PrtP from *Lactobacillus rhamnosus* CGMCC11055. J Agric Food Chem 64:6985-6992.

Gútiez L, Gómez-Sala B, Recio I, del Campo R, Cintas LM, Herranz C, Hernández PE. 2013. *Enterococcus faecalis* strains from food, environmental, and clinical origin produce ACE-inhibitory peptides and other bioactive peptides during growth in bovine skim milk. Int J Food Microbiol 166:93-101.

Hati S, Patel N, Mandal S. 2018. Comparative growth behaviour and biofunctionality of lactic acid bacteria during fermentation of soy milk and bovine milk. Probiotics Antimicrob Proteins 10:277-283.

Hayes M, Stanton C, Slattery H, O'Sullivan O, Hill C, Fitzgerald GF, Ross RP. 2007. Casein fermentate of *Lactobacillus animalis* DPC6134 contains a range of novel propeptide angiotensin-converting enzyme inhibitors. Appl Environ Microbiol 73:4658-4667.

Hebert EM, Mamone G, Picariello G, Raya RR, Savoy G, Ferranti P, Addeo F. 2008. Characterization of the pattern of α_{s1}- and β-casein breakdown and release of a bioactive peptide by a cell envelope proteinase from *Lactobacillus delbrueckii* subsp. *lactis* CRL 581. Appl Environ Microbiol 74:3682-3689.

Hernández-Ledesma B, Amigo L, Ramos M, Recio I. 2004. Angiotensin converting enzyme inhibitory activity in commercial fermented products. Formation of peptides under simulated gastrointestinal digestion. J Agric Food Chem 52:1504-1510.

Ibrahim HR, Ahmed AS, Miyata T. 2017. Novel angiotensin-converting enzyme inhibitory peptides from caseins and whey proteins of goat milk. J Adv Res 8:63-71.

Izquierdo-González JJ, Amil-Ruiz F, Zazzu S, Sánchez-Lucas R, Fuentes-Almagro CA, Rodríguez-Ortega MJ. 2019. Proteomic analysis of goat milk kefir: Profiling the fermentation-time dependent protein digestion and identification of

potential peptides with biological activity. Food Chem 295:456-465.

Jandal JM. 1996. Comparative aspects of goat and sheep milk. Small Rumin Res 22:177-185.

Kalyankar P, Zhu Y, O'Keeffe M, O'Cuinn G, FitzGerald RJ. 2013. Substrate specificity of glutamyl endopeptidase (GE): Hydrolysis studies with a bovine α-casein preparation. Food Chem 136:501-512.

Kim BK, Choi IS, Kim J, Han SH, Suh HJ, Hwang JK. 2017. Effects of fermented milk with mixed strains as a probiotic on the inhibition of loperamide-induced constipation. Korean J Food Sci Anim Resour 37:906-916.

Kunji ER, Mierau I, Hagting A, Poolman B, Konings WN. 1996. The proteolytic systems of lactic acid bacteria. Antonie van Leeuwenhoek 70:187-221.

Li C, Kwok LY, Mi Z, Bala J, Xue J, Yang J, Ma Y, Zhang H, Chen Y. 2017. Characterization of the angiotensin-converting enzyme inhibitory activity of fermented milks produced with *Lactobacillus casei*. J Dairy Sci 100:9495-9507.

Lozo J, Strahinic I, Dalgalarrondo M, Chobert JM, Haertlé T, Topisirovic L. 2011. Comparative analysis of β-casein proteolysis by PrtP proteinase from *Lactobacillus paracasei* subsp. *paracasei* BGHN14, PrtR proteinase from *Lactobacillus rhamnosus* BGT10 and PrtH proteinase from *Lactobacillus helveticus* BGRA43. Int Dairy J 21:863-868.

Lunow D, Kaiser S, Rückriemen J, Pohl C, Henle T. 2015. Tryptophan-containing dipeptides are C-domain selective inhibitors of angiotensin converting enzyme. Food Chem 166:596-602.

Moslehishad M, Ehsani MR, Salami M, Mirdamadi S, Ezzatpanah H, Naslaji AN, Moosavi-Movahedi AA. 2013. The comparative assessment of ACE-inhibitory and antioxidant activities of peptide fractions obtained from fermented camel and bovine milk by *Lactobacillus rhamnosus* PTCC 1637. Int Dairy J 29:82-87.

Nikolić M, Tolinački M, Fira D, Golić N, Topisirović L. 2009. Variation in specificity of the PrtP extracellular proteinases in *Lactococcus lactis* and *Lactobacillus paracasei* subsp. *paracasei*. Folia Microbiol 54:188-194.

Papadimitriou CG, Vafopoulou-Mastrojiannaki A, Silva SV, Gomes AM, Malcata FX, Alichanidis E. 2007. Identification of peptides in traditional and probiotic sheep milk yoghurt with angiotensin I-converting enzyme (ACE)-inhibitory activity. Food Chem 105:647-656.

Parmar H, Hati S, Panchal G, Sakure AA. 2020. Purification and production of novel angiotensin I-converting enzyme (ACE) inhibitory bioactive peptides derived from fermented goat milk. Int J Pept Res Ther 26:997-1011.

Perpetuo EA, Juliano L, Lebrun I. 2003. Biochemical and pharmacological aspects of two bradykinin-potentiating peptides obtained from tryptic hydrolysis of casein. J Protein Chem 22:601-606.

Pihlanto A, Virtanen T, Korhonen H. 2010. Angiotensin I converting enzyme (ACE) inhibitory activity and antihypertensive effect of fermented milk. Int Dairy J 20:3-10.

Qian B, Xing M, Cui L, Deng Y, Xu Y, Huang M, Zhang S. 2011. Antioxidant, antihypertensive, and immunomodulatory activities of peptide fractions from fermented skim milk with *Lactobacillus delbrueckii* ssp. *bulgaricus* LB340. J Dairy Res 78:72-79.

Quirós A, del Mar Contreras M, Ramos M, Amigo L, Recio I. 2009. Stability to gastrointestinal enzymes and structure–activity relationship of β-casein-peptides with antihypertensive properties. Peptides 30:1848-1853.

Rai AK, Sanjukta S, Jeyaram K. 2017. Production of angiotensin I converting enzyme inhibitory (ACE-I) peptides during milk fermentation and their role in reducing hypertension. Crit Rev Food Sci Nutr 57:2789-2800.

Raveschot C, Cudennec B, Deracinois B, Frémont M, Vaeremans M, Dugersuren J, Demberel S, Drider D, Dhulster P, Coutte F, Flahaut C. 2020. Proteolytic activity of *Lactobacillus* strains isolated from Mongolian traditional dairy products: A multiparametric analysis. Food Chem 304:125415.

Rodríguez-Figueroa JC, González-Córdova AF, Torres-Llanez MJ, Garcia HS, Vallejo-Cordoba B. 2012. Novel angiotensin I-converting enzyme inhibitory peptides produced in fermented milk by specific wild *Lactococcus lactis* strains. J Dairy Sci 95:5536-5543.

Rubak YT, Nuraida L, Iswantini D, Prangdimurti E. 2020. Angiotensin-I-converting enzyme inhibitory peptides in milk fermented by indigenous lactic acid bacteria. Vet World 13:345-353.

Seppo L, Jauhiainen T, Poussa T, Korpela R. 2003. A fermented milk high in bioactive peptides has a blood pressure-lowering effect in hypertensive subjects. Am J Clin Nutr 77:326-330.

Shu G, Yang H, Chen H, Zhang Q, Tian Y. 2015. Effect of incubation time, inoculum size, temperature, pasteurization time, goat milk powder and whey powder on ACE inhibitory activity in fermented milk by *L. plantarum* LP69. Acta Sci Pol Technol Aliment 14:107-116.

Solieri L, De Vero L, Tagliazucchi D. 2018. Peptidomic study of casein proteolysis in bovine milk by *Lactobacillus casei* PRA205 and *Lactobacillus rhamnosus* PRA331. Int Dairy J 85:237-246.

Swaisgood HE. 1993. Review and update of casein chemistry. J Dairy Sci 76:3054-3061.

Toe CJ, Foo HL, Loh TC, Mohamad R, Abdul Rahim R, Idrus Z. 2019. Extracellular proteolytic activity and amino acid production by lactic acid bacteria isolated from Malaysian foods. Int J Mol Sci 20:1777.

Torres-Llanez MJ, González-Córdova AF, Hernandez-Mendoza A, Garcia HS, Vallejo-Cordoba B. 2011. Angiotensin-converting enzyme inhibitory activity in Mexican Fresco cheese. J Dairy Sci 94:3794-3800.

Villegas JM, Picariello G, Mamone G, Beatriz M, Turbay E, Giori GS, Hebert EM. 2014. Milk-derived angiotensin-I-converting enzymeinhibitory peptides generated by *Lactobacillus delbrueckii* subsp. *lactis* CRL 581. Peptidomics 1:22-29.

Wang J, Li C, Xue J, Yang J, Zhang Q, Zhang H, Chen Y. 2015. Fermentation characteristics and angiotensin I-converting enzyme-inhibitory activity of *Lactobacillus helveticus* isolate H9 in cow milk, soy milk, and mare milk. J Dairy Sci 98:3655-3664.

WHO. 2021. Cardiovascular diseases (CVDs). Available from: https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds). Accessed at Jan 16, 2021.

Wu J, Aluko RE, Nakai S. 2006. Structural requirements of angiotensin I-converting enzyme inhibitory peptides: Quantitative structure-activity relationship modeling of peptides containing 4-10 amino acid residues. QSAR Comb Sci 25:873-880.

Wu N, Xu W, Liu K, Xia Y, Shuangquan. 2019. Angiotensin-converting enzyme inhibitory peptides from *Lactobacillus delbrueckii* QS306 fermented milk. J Dairy Sci 102:5913-5921.

Zeng Y, Wang N, Qian W. 2013. Production of angiotensin I converting enzyme inhibitory peptides from peanut meal fermented with lactic acid bacteria and facilitated with protease. Adv J Food Sci Technol 5:1198-1203.

Zhao YQ, Zhang L, Tao J, Chi CF, Wang B. 2019. Eight antihypertensive peptides from the protein hydrolysate of Antarctic krill (*Euphausia superba*): Isolation, identification, and activity evaluation on human umbilical vein endothelial cells (HUVECs). Food Res Int 121:197-204.