

# Peptide profiling of goat milk fermented by *Lactobacillus delbrueckii* ssp. *delbrueckii* BD7: Identification of potential biological activity

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**Abstract.** Rubak YT, Nuraida L, Iswanti D, Prangdimurti E, Sanam MUE. 2021. Peptide profiling of goat milk fermented by *Lactobacillus delbrueckii* ssp. *delbrueckii* BD7: Identification of potential biological activity. *Biodiversitas* 22: 3136-3145. This study investigated the angiotensin-converting enzyme (ACE) inhibitory activity in fermented goat milk by *Lactobacillus delbrueckii* ssp. *delbrueckii* BD7, characterizing the peptide and its potential as a bioactive peptide. The starter culture (2%) was inoculated into pasteurized goat skim milk (11%), then incubated at 37 °C until it reached pH 4.6. Centrifugation at 6000 g x 10 minutes at 4 °C was applied. The supernatant obtained was then ultrafiltrated using a membrane cut-off with a molecular weight of 3 kDa, and the fraction obtained was analyzed to determine the inhibitory activity of ACE. Peptides were characterized using Nano LC / MS / MS, and identification as bioactive peptides was carried out based on a literature review. ACE inhibitory activity of fermented goat milk of *Lb. delbrueckii* ssp. *delbrueckii* BD7 was  $55.98 \pm 3.53\%$ . A total of 157 peptides were released with molecular weights ranging from 770.78-2081.12 Da and having 7-19 amino acid residues. The main peptide was hydrolyzed from casein (72.6%), cleavage in the parent protein, specific for aliphatic and aromatic amino acids. Identification of bioactive peptides based on the similarity of amino acid residues at C-terminal obtained 28 ACE inhibitor peptides, 19 antioxidant peptides, and ten antimicrobial peptides. Some of these peptides have homologous sequences with previously reported peptides. *Lb. delbrueckii* ssp. *delbrueckii* BD7 has the potential as a starter culture to produce fermented milk, which is rich in biological activity.

**Keywords:** Angiotensin, bioactive peptides, fermented goat milk, Lactic acid bacteria, *Lb. delbrueckii* ssp. *delbrueckii* BD7

## INTRODUCTION

Bioactive peptides have been defined as certain parts or fragments of encrypted proteins in the primary protein sequence and positively impact the function or condition of the body to affect the overall health status of the human body. These health benefits have been linked to various biological activities of bioactive peptides in the cardiovascular, digestive, immune, and nervous systems (Li et al. 2019; Daliri et al. 2017). Bioactive peptides have several characteristics in terms of their order and molecular weight. The molecular weight of bioactive peptides is reported to be <10 kDa, with amino acid residues of 2 to 20 (Li et al. 2017; Kim et al. 2016). Another characteristic that bioactive peptides have is specific amino acid residues at the C-terminal (Abdel-Hamid et al. 2017). Aliphatic and aromatic amino acids are the dominant amino acids in the C-terminal of a bioactive peptide. Proline, Arginine, and Lysine are the dominant amino acids in the C-terminal of ACE inhibitor peptides (Rai et al. 2017). Likewise, those

found in antimicrobial bioactive peptides, antioxidants, and immunomodulatory peptides.

Foods with high protein content are known to be a source of bioactive peptides. Milk and dairy products, including fermented milk, have been identified as sources of bioactive peptides. The documentation of peptides produced from fermented milk includes immunomodulatory peptides (Zhao et al. 2014), anticancer (Ayyash et al. 2018; Elfahri et al. 2016), antimicrobial (Algboory et al. 2018; Retnaningrum et al. 2020), binding minerals (Zhao et al. 2015; Gaetano-Silva et al. 2015), antihypertensives and antioxidants (Rana et al. 2018; Georgalaki et al. 2017). Fermentation is an exciting approach to produce bioactive peptides from milk protein using microbes. Lactic acid bacteria (LAB) are the dominant bacteria involved during the fermentation process. During fermentation, LAB will actively hydrolyze proteins into amino acids and peptides for their growth needs. The peptides released vary in number and sequence of peptides, and among them are bioactive peptides. The release of these bioactive peptides in various fermented

foods is strain-specific (Li et al. 2017; Wang et al. 2015). Protein hydrolysis by LAB involves a proteolytic system. One of the keys to protein hydrolysis in LAB proteolytic system is the Cell Envelope Protease (CEP). The components of CEP differ between LAB and can even be different in one species (Lozo et al. 2011; Raveschot et al. 2020), which impacts the specificity of cleavage in parent protein. CEP's difference has clearly explained why the peptides released varied both in number and sequence among LAB.

Certain LAB has been known to release bioactive peptides. Several strains of *Lactobacillus* and *Lactococcus* have been used as starter cultures to produce bioactive peptides (Chen et al. 2015; Barla et al. 2016). Cultures can be used alone or with other LAB (Chaves-López et al. 2014). The potential of LAB will continue to be explored because of the increasing human awareness of health. Through exploration, certain LAB strains can be obtained, which can be applied to produce functional food products that provide nutritional components and specific biological activities for the human body. This study aims to determine the angiotensin-converting enzyme's inhibitory activity in fermented goat milk by *Lactobacillus delbrueckii* ssp. *delbrueckii* BD7 characterized the resulting peptides and their potential as bioactive peptides. *Lb. delbrueckii* ssp. *delbrueckii* BD7 was isolated from kefir and had a high proteolytic activity based on our previous study results (Rubak et al. 2020).

## MATERIAL AND METHODS

### Culture

*Lactobacillus delbrueckii* ssp. *delbrueckii* BD7 was obtained from Southeast Asian Food and Agricultural Science and Technology (SEAFST) Center, IPB University (Bogor Agricultural University), Bogor, Indonesia. LAB stock cultures were freshened in De Man Rogosa and Sharpe (MRS) broth (Oxoid, USA) and incubated at 37°C for 24 hours. 2% of LAB in MRS broth was transferred into reconstituted goat milk and incubated at 37°C for 24 h. This adaptation stage was carried out twice.

### Fermentation of goat milk

The starter culture (2%) was inoculated into pasteurized goat skim milk (11%, Sukamilk, Dutch) and then incubated at 37 ° C until pH 4.6 was reached (700 Eutech). Fermentation was stopped by heating at 75 ° C for 1 minute; then fermented goat milk was centrifuged (Hettich, Zentrifugen, Mikro 22R) at 6000 gx 10 minutes at 4 ° C. The supernatant was collected, followed by ultrafiltration using a molecular weight membrane cut of 3 kDa (Merck, Amicon Ultra-4 mL, Centrifugal Filter, IRL). The supernatant was put into a filter tube and centrifuged at 4000 xg, 30 minutes, 4 ° C. Fractions were collected to determine ACE inhibitory activity (Chusman 1971).

### In vitro assay for inhibitory activity

Hippuryl-L-Histidyl-L-Leucine (HHL, Sigma, USA) was used as an enzyme substrate. A total of 50 µL of the sample was mixed with 50 µL of substrate [50 mM HHL in 0.1 M sodium borate buffer (pH 8.3) containing 0.3 M NaCl] and incubated at 37 ° C for 5 min. A total of 50 µL of 0.1 U mL<sup>-1</sup> ACE solution (Rabbit lung, Sigma, USA) was added, then incubated at 37 ° C for 5 min. 250 µL of 1 M HCl solution was added to stop the reaction. Then, 1.5 mL of ethyl acetate was added to extract the hippuric acid (HA) released by ACE from the reaction mixture using vortex mixing for 10 s followed by centrifugation at 2000 × g for 5 min. The 0.8 mL ethyl acetate layer was transferred to a clean tube and evaporated at 85 ° C for 60 min. The HA in the tube was dissolved in 4 mL of distilled water and cooled to room temperature. At an optical density of 228 nm, the amount of HA formed was measured (UV-2800, Hitachi, JPN). The inhibition rate was calculated as 100% [(B-A) / B], where A is the optical density in the presence of ACE and ACE inhibitor components, and B is the optical density without the ACE inhibitor component.

### Identification of peptides by mass spectrometry

Identification of the peptides was done using the liquid chromatography-mass spectrometry (LC-MS), Nano LC Ultimate 3000 series system Tandem Q Exactive Plus Orbitrap HRMS (Thermo scientific, GER), with 5 µL sample, a Thermo scientific trap column (164649, 30 µm x 5 mm) and an eluent of 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 for 6 min. The peptides were 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 the final step with solvent B (90%) over 5 minutes to solvent B (5%) over 5 minutes. Electrospray was carried out at an ion spray voltage of 3500 eV. The MS/MS spectrum was collected from 200 to 2000 m/z. Through Proteomic Discoverer software version 2.2, peptides in the sample are identified.

## RESULT AND DISCUSSION

### ACE inhibitory activity in fraction separated according to molecular weight by ultrafiltration

Two fractions namely fraction <3 kDa and > 3 kDa were obtained from the ultrafiltration of goat's milk supernatant fermented by *Lb. delbrueckii* ssp. *delbrueckii* BD7 uses 3 kDa Molecular Weight Cut Off (MWCO). Measurement of ACE inhibitory activity in the two fractions is presented in Table 1. The results showed that the ACE inhibitory activity in the <3 kDa fraction (55.98 ± 3.53%) was higher than the > 3 kDa fraction.

The results obtained reaffirmed that the highest ACE inhibitory activity was generally in the <3 kDa fraction as reported by previous researchers (Fan et al. 2018; Moreno-Montoro et al. 2018). Several researchers have reported

*Delbrueckii* species producing ACE inhibitory activity, with activity above 50%. *Lb. delbrueckii* subsp. *bulgaricus* 92059 (Li et al. 2019), *Lb. delbrueckii* subsp. *bulgaricus* ACA-DC 87 (Georgalaki et al. 2017), *Lb. delbrueckii* QS306 (Wu et al. 2019) and *Lb. bulgaricus* LB6 (Shu et al. 2019). A previous study by (Qian et al. 2011) and (Villegas et al. 2015) reported ACE inhibitory activity of *Lb. delbrueckii* ssp. *bulgaricus* LB340 and *Lb. delbrueckii* subsp. *lactis* CRL 58. In addition to being strain-specific, the use of substrates also affects ACE inhibitory activity (Wang et al. 2016; Georgalaki et al. 2017). Several researchers have reported that goat milk has ACE inhibitory activity (Ibrahim et al. 2017; Izquierdo-González et al. 2019).

### Identification of peptides generated from goat milk fermented by *Lb. delbrueckii* ssp. *delbrueckii* BD7 in the <3 kDa fraction

During fermentation, through its proteolytic system, LAB actively hydrolyzes proteins into peptides and free amino acids, some of which will be brought into the cell as a source of nitrogen for metabolism. Some peptides will accumulate on the substrate (Fan et al. 2019). A total of 157 peptides (data not presented) were released in fermented goat milk by *Lb. delbrueckii* ssp. *delbrueckii* BD7 (<3 kDa), during incubation 48 h at 37 °C. Most of peptides were hydrolyzed from casein as much as 72.6% ( $\alpha$ S1-casein 9.6%,  $\alpha$ S2-casein 14.6%,  $\beta$ -casein 43.3%, and  $\kappa$ -casein 5.1%), others were hydrolyzed from whey as much 32.4% ( $\alpha$ -lactalbumin 1.9%,  $\beta$ -lactoglobulin 3.8%, and serum amyloid A 21.7%). Peptide profile of fermented goat milk by *Lb. delbrueckii* ssp. *delbrueckii* BD7 is summarized in Table 2. The amount of hydrolyzed peptides in this study was seen at the chromatogram peak using Nano LC / MS.MS (Figure 1).

The protein in goat milk consists of casein (80%) and whey (20%). Casein is a major source of amino acids and peptides (Karami et al. 2019).  $\beta$ -casein is the dominant parent protein in goat milk, about 54.8% of total casein (Selvaggi et al. 2014). Most of the peptides released by *Lb. delbrueckii* ssp. *delbrueckii* BD7 in fermented goat milk

are derived from the cleavage of the parent protein  $\beta$ -casein (Table 2). Similar results have been reported in the studies of Ali et al. (2019) and Kliche et al. (2017). The flexible and open structure of  $\beta$ -casein facilitates the accessibility of CEP *Lb. delbrueckii* ssp. *delbrueckii* BD7. Additionally, Ji et al. (2021) stated that the CEP properties of *Lb. delbrueckii* is a zinc-dependent monomer ~70kDa, which degrades intact casein with a significant preference for  $\beta$ -casein. However, during fermentation, peptides could also be hydrolyzed from other protein parents in relatively small amounts. The number of peptides released from each parent protein varied, highly strain-dependent. Not all *Lb. delbrueckii* is able to hydrolyze casein. Kliche et al. (2017) reported that *Lb. delbrueckii* ssp. *bulgaricus* 92059 could not hydrolyze  $\kappa$ -casein. Proteolytic activity determines the ability of protein hydrolysis by LAB. Lactic Acid Bacteria have a complex proteolytic system, which involves at least three main components: CEP, specific transporters, and intracellular peptidase (Savijoki et al. 2006). The LAB proteolytic system's complexity is indicated by the parent protein specificity, which is cleaved to produce various peptides from milk protein.

The peptide released by *Lb. delbrueckii* ssp. *delbrueckii* BD 7 has a molecular weight of 770.78-2081.12 Da, with the shortest peptide length consisting of 7 amino acids ( $\beta$ -casein,  $\kappa$ -casein,  $\alpha$ -lactalbumin, and serum amyloid A) and the longest with 19 amino acid residues ( $\alpha$ s1-casein). The peptide length identified from *Lb. delbrueckii* could consist of 3 to 20 amino acids (Villegas et al. 2014).

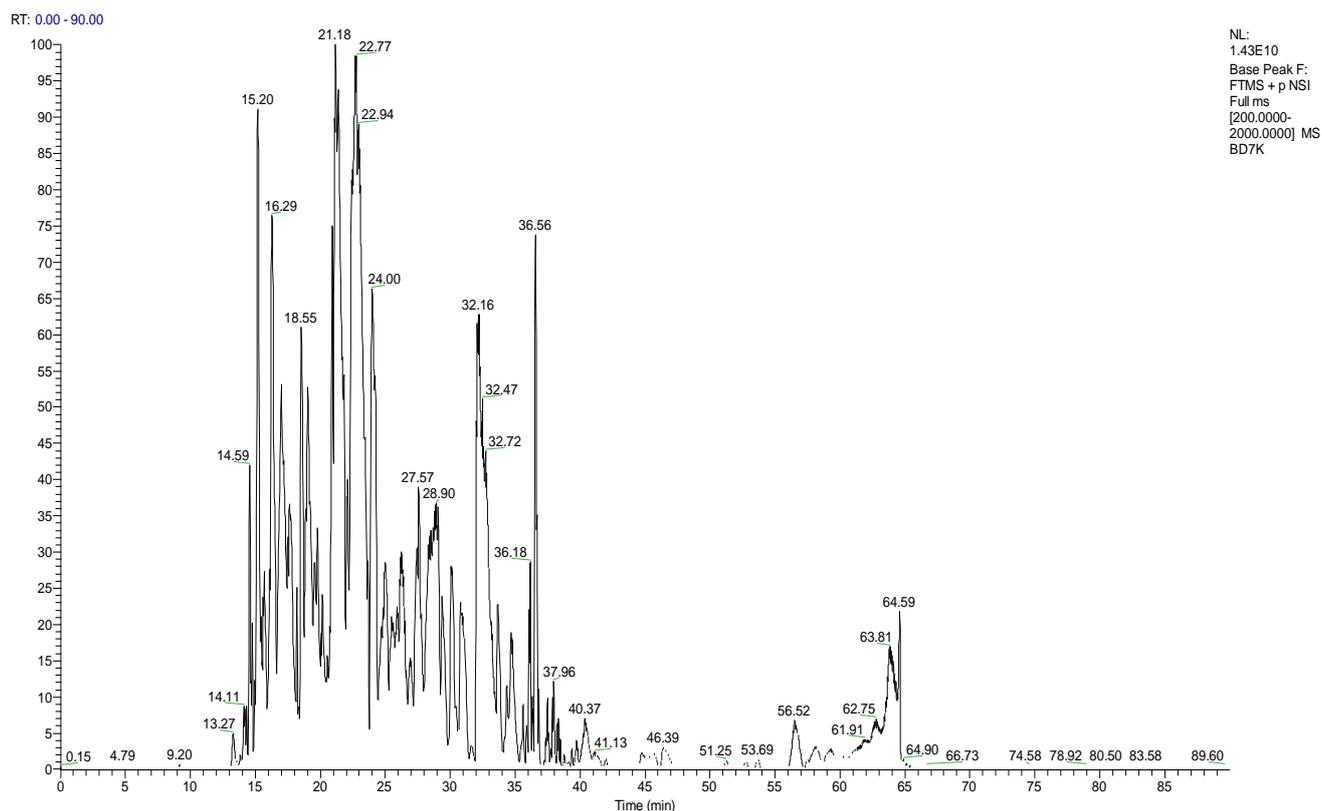
**Table 1.** Angiotensin-converting enzyme inhibitory activity in fractions > 3kDa and <3 kDa of fermented goat milk by *Lb. delbrueckii* ssp. *delbrueckii* BD7

Lactic acid bacteria	ACE inhibitory activity (%)	
	>3 kDa	<3 kDa
<i>Lb. delbrueckii</i> ssp. <i>delbrueckii</i> BD7	28.81 ± 1.87	55.98 ± 3.53

**Table 2.** Peptide profiles of <3 kDa fraction of fermented goat milk of *Lb. delbrueckii* ssp. *delbrueckii* BD7

Parent protein*	Total peptides	Amino acid residue	MH+ [Da]	m/z [Da]	Dominant peptide
$\alpha$ s1-casein	15	7-19	940.47-1844.86	940.47-1844.85	FSDIPNPIGSENSGKTTMP
$\alpha$ s2-casein	23	8-17	990.48-2081.12	990.48-2081.10	ITVDDKHY
$\beta$ -casein	68	7-16	770.48-2081.12	770.47-1966.88	QQQTEDELQDKIHP, TEDELQDKIHP
$\kappa$ -casein	8	7-11	803.44-1268.65	400.20-634.83	FLPYPPY
$\alpha$ -lactalbumin	3	7-10	905.43-1126.47	905.43-1126.47	FHTSGYDTQA
$\beta$ -lactoglobulin	6	8-11	903.56-1270.69	903.55-1270.68	TKIPAVFK
Serum amyloid A	34	7-17	833.47-1938.93	833.47-1938.91	ITDPLFKG

Note: A: Alanine, D: Aspartic Acid, E: Glutamic Acid, F: Phenylalanine, G: Glycine, I: Isoleucine, K: Lysine, L: Leucine, M: Methionine, N: Asparagine, P: Proline, Q: Glutamine, R: Arginine, S: Serine, T: Threonine, V: Valine, W: Tryptophan, Y: Tyrosine. \*Protein access code at <https://www.uniprot.org/>.

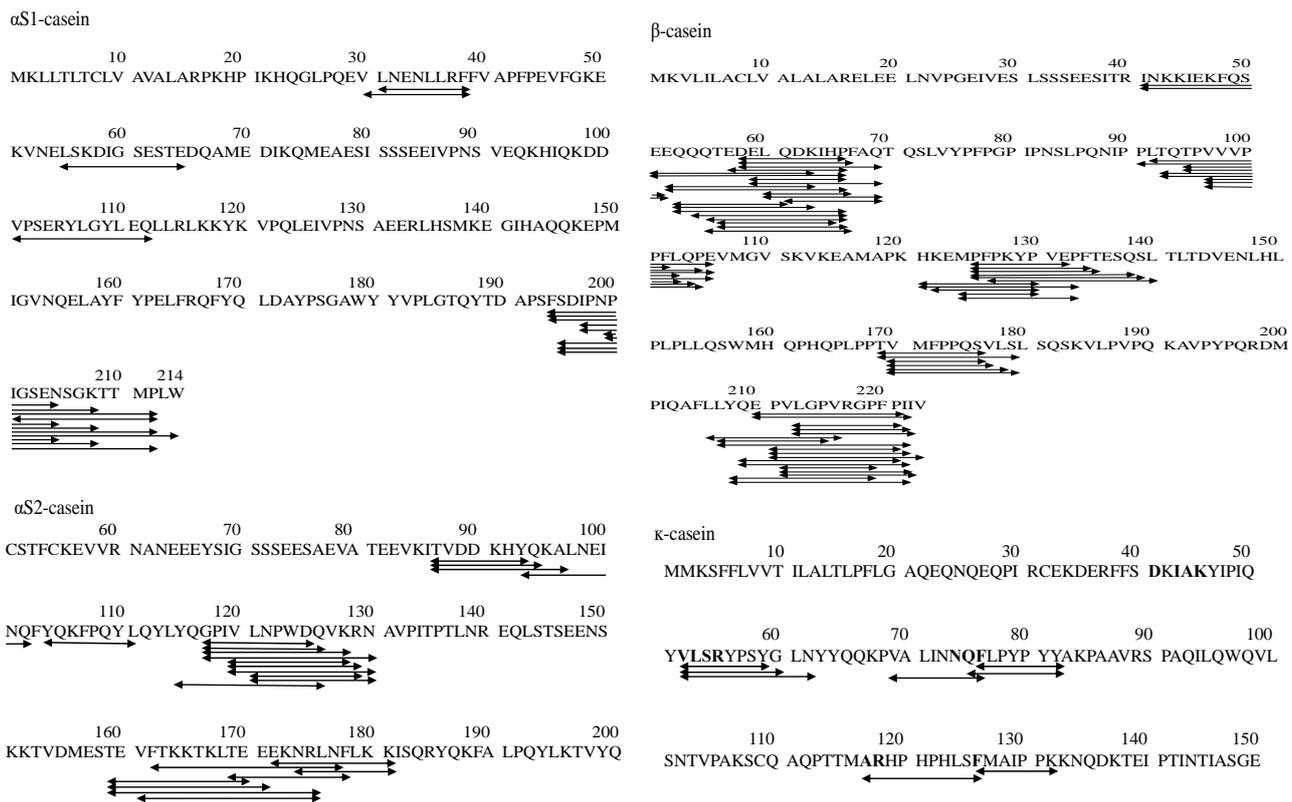


**Figure 1.** Nano LC-MS/MS mass spectra of goat milk fermented by *Lb. delbrueckii* ssp. *delbrueckii* BD7 in a mass range from  $m/z$  200-2000

Some researchers stated that fermentation conditions such as temperature and fermentation time could also affect the peptide variations produced by LAB (Chen et al. 2015; Li et al. 2017; Shu et al. 2015). LAB growth conditioning under optimal conditions will result in maximum protein hydrolysis. In our study, goat milk fermentation was carried out at 37 °C, and fermentation was stopped when pH 4.6 was reached. *Lactobacillus delbrueckii* had a growth temperature variation between 37-42 °C. In the research of Villegas *et al.*, 2014, *Lb. delbrueckii* subsp. *lactis* CRL 58 grew well at 40 °C, others grow at 37 °C (Georgalaki et al. 2017; Shu et al. 2018) and 42 °C (Shi et al. 2017). The pH during fermentation is also a concern. High acidification is thought to inhibit or stop the protein hydrolysis process so that some researchers then stop fermentation when pH 4.5 has been reached (Chen et al. 2015). This is related to the sensitivity of the protease to acidification.

Cell envelope proteinase is an extracellular proteolytic enzyme from LAB responsible for the first step in the hydrolysis of casein to produce peptides and amino acids. Cell envelope proteinase has been characterized in several LAB. Kunji (1996) divided CEP of LAB into three types based on the substrate being hydrolyzed, namely 1) CEP type PI, which hydrolyzes  $\beta$ -casein explicitly, 2) CEP type PIII, which hydrolyzes  $\alpha$ S1-casein and  $\kappa$ -casein, 3) CEP type PI and PIII, hydrolyzes apart from  $\beta$ -casein also  $\alpha$ S1-

casein. Based on the results obtained in this study, the dominant peptide hydrolysis is produced from  $\beta$ -casein so that the CEP type from *Lb. delbrueckii* ssp. *delbrueckii* BD 7 is categorized into type P1 and type P1 / PIII because there are peptides released from other parent proteins. Hebert et al. (2008) previously reported the CEP type of *Lb. delbrueckii* subsp. *lactis* CRL 581 is a PI type and PI / PIII intermediate type. The specificity of *Lb. delbrueckii* ssp. *delbrueckii* BD7 cleavage in the parent protein was evaluated for casein protein (Figure 2). In the parent protein  $\alpha$ S1-casein, the dominant peptide is hydrolyzed by cleaving the amino acids Serine and Phenylalanine (S193-F194, F194-S195), on  $\alpha$ S2-casein, the amino acids Isoleucine, Glutamine, and Serine (I86-T87, I119-V120, Q116-G117), whereas for  $\beta$ -casein, the cleavage specificity at amino acids glutamate, Proline and Methionine (M124-P125, T169-V170, P95-V96, P211-V212) and on  $\kappa$ -casein on the amino acid Tyrosine (Y51-V52). It appears that cleavage of the parent protein occurs in certain amino acids, and it appears to be the specificity of cleavage *Lb. delbrueckii* ssp. *delbrueckii* BD7 in casein is dominant for nonpolar/aliphatic amino acids and uncharged polar amino acids. This study's results are also in line with those reported by Hebert et al. (2008) that the cleavage specificity of *Lb. delbrueckii* is dominant in hydrophobic or aromatic amino acids.



**Figure 2.** Specific cleavage of the parent proteins  $\alpha$ s1-casein,  $\alpha$ s2-casein,  $\beta$ -casein, and  $\kappa$ -casein by CEP from *Lb. delbrueckii* ssp. *delbrueckii* BD7 to produce a number of peptides

### ACE-inhibitors peptides and other bioactive peptides in the <3 kDa fraction of goat milk fermented by *Lb. delbrueckii* ssp. *delbrueckii* BD7

The ACE inhibitors peptide potential of *Lb. delbrueckii* ssp. *delbrueckii* BD7 was investigated based on a literature search. Several researchers have previously used this method to reveal the potential of peptides as bioactive peptides (Dallas et al. 2016; Georgalaki et al. 2017). In our study, peptides categorized as ACE-inhibitor peptides have similarities (100%) in amino acid residues in the C-terminal of the ACE inhibitor peptides. A total of 28 peptides were identified as ACE inhibitor peptides. Six of them were homologous with reported ACE inhibitor peptides namely MPFPKYPVEP ( $\beta$ -casein, f124-f133), LGPVRGPF (beta-casein, f213-221), DELQDKIHPF ( $\beta$ -casein, f58-f67), YQEPVLGPVRGPFPI ( $\beta$ -casein, f208-f222), QEPVLGPVRGPF (beta-casein, f209-f221), and VLGVRGPF (beta-casein, f212-f221). It was reported that the ACE inhibitor peptide is a specific peptide, which can be identified by the presence of certain amino acids in the C-terminal. The inhibitor's binding to the ACE is strongly influenced by the hydrophobicity of the amino acid residues at the C-terminal. ACE has three active sub-sites, namely S1 (antepenultimate), S1' (penultimate), and S2 (ultimate). These three active sites have different characters in binding amino acid residues at the C-terminal of a peptide.

The dominant amino acid residues occupy positions one, two, or three on the C-terminal of *Lb. delbrueckii* ssp. *delbrueckii* BD7 released peptides are the amino acids Proline, Lysine, and Phenylalanine. ACE prefers competitive substrates or inhibitors containing hydrophobic amino acids such as Proline, Lysine, and Arginine (Gútiérrez et al. 2013; Aslam et al. 2019). Proline is the predominant amino acid present in the C-terminal ACE-inhibitor peptide (Lu et al. 2016; Gonzalez-Gonzalez et al. 2013). In our results, 72 of the 157 peptides had Proline amino acid residues at the C-terminal. Therefore, other peptides also have the potential as ACE inhibitor peptides. Moreover, in our results, it is known that the dominant peptide having Proline amino acid residues at the C-terminal were derived from the parent protein  $\beta$ -casein, so it is not surprising that most of the ACE-inhibitor peptides were derived from  $\beta$ -casein. In contrast, as shown in Table 3, none of the ACE-inhibitor peptides were derived from whey protein ( $\alpha$ -lactalbumin,  $\beta$ -lactoglobulin, and serum amyloid A). Our identification of amino acid residues on the C-terminal peptide released from whey protein indicates that this position is dominated by the amino acids Asparagine, Tyrosine, and Lysine. These amino acids tend to be hydrophilic. Peptides in the presence of hydrophilic amino acids at the C-terminal have not been widely reported to act as ACE inhibitor peptides. However, whey is also a great source of ACE inhibitor peptides (Guo et al. 2019; Ibrahim et al. 2017).

**Table 3.** ACE inhibitor peptides, antioxidant peptides, and antimicrobial peptides identified in fermented goat milk by *Lb. delbrueckii* ssp. *delbrueckii* BD7 through literature search. Residues in bold letters indicate sequence homology

Parent protein*	Previously described sequence	Sequence	MH+ [Da]	m/z [Da]	Activity	References
αs1-CN	<b>FSDIPNPIGSEN</b>	<b>FSDIPNPIGSEN</b>	1289.59	645.30	antioxidant	Hayes et al. (2006) Rana et al. (2018)
αs1-CN	<b>SDIPNPIGSEN</b>	<b>SDIPNPIGSEN</b>	1142.53	571.77	Antioxidant Antimicrobial,	Hayes et al. (2006) Rana et al. (2018)
αs1-CN		<b>IPNPIGSEN</b>	940.47	470.74	antioxidant	
αs1-CN	IGSENSEKTTMP	<b>FSDIPNPIGSENSGKTTMP</b>	1991.92	996.46	ACE inhibitory	Hayes et al. (2007)
αs1-CN		<b>PIGSENSGKTTMP</b>	1318.62	659.81		
αs1-CN		<b>IGSENSGKTTMP</b>	1237.57	619.29		
αs1-CN	TTMPLW	<b>PIGSENSGKTTMPLW</b>	1617.79	809.40	ACE inhibitory	Pihlanto-Leppälä et al. (1998)
αs2-CN	TKKTKLTEEEKNRL	<b>FTKTKLTEEEKNRLN</b>	1979.08	495.52	ACE inhibitory	Srinivas and Prakash (2010)
αs2-CN	LYQGPIVLNPDQVK	<b>GPIVLNPDQVK</b>	1365.74	683.37	ACE inhibitory	Nongonierma et al. (2017)
β-CN	DKIHFP	<b>LQDKIHFP</b>	997.54	499.27	ACE inhibitory	Ibrahim et al. (2017)
β-CN	<b>DELQDKIHFP</b>	<b>DELQDKIHFP</b>	1241.61	621.31	ACE inhibitory	Fan et al. (2018)
β-CN	ELQDKIHFP	<b>TEDELQDKIHFP</b>	1471.70	736.35	ACE inhibitory	Gobbetti et al. (2000)
β-CN	LQDKIHP	<b>DELQDKIHP</b>	1094.54	547.77	antioxidant	Ahmed et al. (2015)
β-CN	DKIHP	<b>EDELQDKIHP</b>	1223.58	612.29	ACE inhibitory	Gobbetti et al. (2000)
β-CN		<b>EEQQTEDELQDKIHP</b>	1966.89	656.30		
β-CN		<b>ELQDKIHP</b>	979.52	490.26		
β-CN	DKIHFFAQ	<b>LQDKIHFFAQ</b>	1196.63	598.82	ACE inhibitory	Papadimitriou et al. (2007)
β-CN		<b>ELQDKIHFFAQ</b>	1325.68	663.34		
β-CN	YVPEPF	<b>FPKYVPEPF</b>	1123.57	562.29	antioxidant	Li et al. (2013)
β-CN	<b>MPFPKYVPEP</b>	<b>MPFPKYVPEP</b>	1204.60	602.80	ACE inhibitory	Hayes et al. (2007)
β-CN		<b>HKEMPFKYVPEP</b>	1598.79	533.60		
β-CN		<b>EMPFKYVPEP</b>	1349.63	675.32		
β-CN	LYQEPVLPVVRGPFPIIV	<b>PVLGPVVRGPFPIIV</b>	1460.89	730.95	ACE inhibitory, antioxidant	Birkemo et al. (2009)
β-CN	QEPVLPVVRGPFPIIV				ACE inhibitory	Zhao et al. (2019)
β-CN	YQEPVLPVVRGPFPIIV				ACE inhibitory antioxidant	Corrons et al. (2017) Eisele et al. (2013)
β-CN	GPVVRGPFPIIV				antimicrobial ACE inhibitory	Birkemo et al. (2009) Contreras et al. (2009)
β-CN	LGPVVRGPFPIIV				antioxidant	Padghan et al. (2017)
β-CN	YQEPVLPVVRGPFPII	<b>QEPVLPVVRGPFPII</b>	1618.93	809.97	antimicrobial	Rana et al. (2018)
β-CN		<b>EPVLPVVRGPFPII</b>	1490.86	745.93	antimicrobial	Birkemo et al. (2009)
β-CN	<b>VLGPVVRGPFPII</b>	<b>VLGPVVRGPFPII</b>	1264.77	632.89	antimicrobial	Rana et al. (2018)
β-CN	GVRGPFPII				antioxidant	Farvin et al. (2010)
β-CN	<b>YQEPVLPVVRGPFPI</b>	<b>YQEPVLPVVRGPFPI</b>	1668.90	834.95	ACE inhibitory antimicrobial	Torres-Llanez et al. (2011) Rana et al. (2018)
β-CN		<b>LYQEPVLPVVRGPFPI</b>	1781.98	891.50		
β-CN	GPVVRGPFPI	<b>EPVLPVVRGPFPI</b>	1377.78	689.39	ACE inhibitory	Hernandez-Ledesma et al. (2004)
β-CN	<b>QEPVLPVVRGPFPI</b>	<b>QEPVLPVVRGPFPI</b>	1392.76	696.88	ACE inhibitory	Villegas et al. (2014)
β-CN	<b>EPVLPVVRGPFPI</b>	<b>EPVLPVVRGPFPI</b>	1264.70	632.85	Antioxidant ACE inhibitory	Padghan et al. (2017) Hayes et al. (2007)
β-CN	<b>VLGPVVRGPFPI</b>	<b>VLGPVVRGPFPI</b>	1555.82	778.41		
β-CN		<b>YQEPVLPVVRGPFPI</b>	1038.60	519.80	antimicrobial ACE inhibitory	Quirós et al. (2007) Gútiérrez et al. (2013)
β-CN	<b>LGPVVRGPFPI</b>	<b>LGPVVRGPFPI</b>	939.53	470.27	ACE inhibitory	Villegas et al. (2014)
β-CN	<b>VVVPFLQP</b>	<b>VVVPFLQP</b>	995.59	498.30	antimicrobial	Rana et al. (2018)
β-CN	LTQTPVVVPFL	<b>TQTPVVVPFL</b>	1197.68	599.34	antimicrobial	(Rana et al. (2018)
β-CN	LTQTPVVVPF	<b>PLTQTPVVVPF</b>	1294.73	647.87	ACE inhibitory	Quirós et al. (2009)
κ-CN	HPHPLSF	<b>ARHPHPLSF</b>	1198.61	400.21	antioxidant	Shanmugam et al. (2015)

Note: CN: Casein, A: Alanine, D: Aspartic Acid, E: Glutamic Acid, F: Phenylalanine, G: Glycine, I: Isoleucine, K: Lysine, L: Leucine, M: Methionine, N: Asparagine, P: Proline, Q: Glutamine, R: Arginine, S: Serine, T: Threonine, V: Valine, W: Tryptophan, Y: Tyrosine.  
\*Protein access code at <https://www.uniprot.org/>.

Additionally, peptide length is also known as a characteristic of ACE inhibitor peptides. ACE inhibitor peptides are known to consist of 3 to 20 amino acids with molecular weights <3kDa (Lu et al. 2016; Aslam et al. 2019). In our study, peptides with potential as ACE inhibitor peptides consisted of 8 to 19 amino acids. Short peptides are known to bind to active ACE sites easily. Short peptides with certain C-terminal amino acid residues are known to be more resistant to gastrointestinal hydrolysis. Therefore, they do not lose their ability as ACE inhibitor peptides after oral administration (Contreras et al. 2013). In addition, short peptides are thought to be more easily transported through intestinal cells to reach the cardiovascular system. Fujita et al. (2000) have been classified ACE inhibitor peptides into three types: a) Type I, true inhibitor, in which the IC<sub>50</sub> of the peptide is not affected by gastrointestinal protease or preincubation with ACE, b) Type II: Substrate type, represented by ACE inhibitors by gastrointestinal proteases, and c) Type III: pro-drug, which is peptides that are converted to potential ACE inhibitors by the action of gastrointestinal proteases (Wang et al. 2020). It is expected that the peptides produced by a starter culture are true inhibitors or pro-drugs. Several ACE inhibitor peptides type I and III have been reported by Liu and Pischetsrieder (2017) and Quirós et al. (2009).

The peptides released in fermented milk could have more than one biological activity (Qian et al. 2011; Moreno-Montoro et al. 2018). In our study, the presence of other bioactive peptides released by *Lb. delbrueckii* ssp. *delbrueckii* BD7 during fermentation was also identified, namely antioxidant peptides and antimicrobial peptides. The presence of antioxidant peptides in fermented foods has also attracted much attention. Increased susceptibility to disease has been associated with increased production of reactive oxygen species in plasma and cells (Guo et al. 2014). Peptides that act as antioxidant peptides could interact with radical species or inhibit oxidative reactions. Panchal et al. (2020) and Shu et al. (2018) reported the antioxidant activity in fermented foods, especially in fermented goat milk. In our study, a total of 19 peptides were identified as antioxidant peptides (Table 3). Four of them have 100% homology with antioxidant peptides that have been reported by several researchers, namely FSDIPNPIGSEN ( $\alpha$ S1-casein, f194-f205), SDIPNPIGSEN ( $\alpha$ S1-casein, f195-f205), EPVLGPVGRPFPI ( $\beta$ -casein, f210-f221), and LGPVGRPFPII ( $\beta$ -casein, f213-f223). Similar to other bioactive peptides, antioxidant peptides are also characterized by their sequence and composition. Hydrophobic and aromatic amino acid residues in peptides such as the amino acids Tyrosine, Tryptophan, Methionine, and Lysine have been associated with antioxidant activity (Matsui et al. 2018). These amino acid residues were also identified in the peptide sequence released by *Lb. delbrueckii* ssp. *delbrueckii* BD7, predominantly present in peptides released from the parent proteins  $\beta$ -lactoglobulin (Tyrosine and Lysine),  $\kappa$ -casein, and  $\alpha$ S2-casein (Lysine). Despite our identification results (Table 3), most antioxidant peptides were released from  $\beta$ -casein. However, identifying the presence of a specific amino acid

in the peptide sequence can be used as a guide in determining potential peptides as antioxidant peptides.

The presence of antimicrobial peptides in our results indicates that a total of 10 peptides were identified as antimicrobial peptides. Three of them have homologous sequences with reported antimicrobial peptides (Rana et al. 2018), namely SDIPNPIGSEN ( $\alpha$ S1-casein, f195-f205), VLGPRVGRPFPI ( $\beta$ -casein, f212-f221), and VVVPPFLQP ( $\beta$ -casein, f97-f105). The presence of these peptides indicates that antimicrobial activity can be produced in fermented goat milk, in line with the results reported by Biadała et al. (2020) in goat milk fermented by *Lb. delbrueckii* subsp. *lactis* PCM 2611 and in the research of Rana et al. (2018). Antimicrobial peptides have also been identified as short peptides. The presence of amino acid residues Glycine, Leucine, and Arginine in peptide sequences play a vital role in antimicrobial activity (Sahariah and Måsson 2017; Gagnon et al. 2017).

Interestingly, from our identification results, several peptides had more than one biological activity (Table 3), namely the peptide SDIPNPIGSEN (Antioxidant and antimicrobial;  $\alpha$ S1-casein, f195-f205), YQEPVLGPVGRPFPI (ACE inhibitor and antimicrobial;  $\beta$ -casein, f208-f222), VLGPRVGRPFPI (ACE inhibitor and antioxidant;  $\beta$ -casein, f212-f221), and EPVLGPVGRPFPI (ACE inhibitor and antioxidant;  $\beta$ -casein, f210-f221). These peptides have great uses because they can simultaneously trigger, modulate, or inhibit several physiological pathways. In the body system, it is observed that bioactivity in one area often produces beneficial effects in another (Sistla 2013; Agyei and He 2015). Peptides that have more than one biological activity have also previously been reported in research by Moreno-Montoro et al. (2018), Taha et al. (2017), and Sah et al. (2014). In particular, it appears from our identification results that the identified bioactive peptides are predominantly released from casein hydrolysis. Bioactive peptides can also be produced from whey protein hydrolysis (Dullius et al. 2018; de Lima et al. 2018). However, not many studies have demonstrated the bioactive peptide sequence of whey protein. Some of the reported peptides from whey protein do not match the peptide sequences produced in our study.

In conclusion, ACE inhibitory activity was produced in goat milk fermented by *Lb. delbrueckii* ssp. *delbrueckii* BD7. A total of 157 peptides were produced during incubation 48 h at 37 °C. These peptides have a molecular weight ranging from 770.78 to 2081.12 Da and consist of 7-19 amino acids. The main peptide was hydrolyzed from casein (72.6%). Cell envelope proteinase of *Lb. delbrueckii* ssp. *delbrueckii* BD7 cleavage on the parent protein, specific for aliphatic and aromatic amino acids. A total of 28 ACE inhibitor peptides, 19 antioxidant peptides, and ten antimicrobial peptides were identified. Several peptides have homologous sequence with the bioactive peptides that have been reported. This study has shown ACE peptides and other bioactive peptides in goat's milk fermented by *Lb. delbrueckii* ssp. *delbrueckii* BD7. These provide an understanding that fermented goat milk is rich in benefits from its nutritional content and the biological activity of peptides produced during fermentation. Another thing is

that using a single isolate does not reduce the benefits obtained from fermented milk. The choice of strain affects the effective release of bioactive peptides. The strain must have the right specificity to produce several bioactive peptides. *Lb. delbrueckii* ssp. *delbrueckii* BD7 has the potential as a starter culture to produce fermented milk, which is rich in biological activity.

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