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PRODUCTION OF ANTIHYPERTENSIVE BIOACTIVE PEPTIDES IN FERMENTED FOOD BY LACTIC ACID BACTERIA – A REVIEW

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ABSTRACT
Antihypertensive bioactive peptides are one of the natural sources that can be used in preventing hypertension. Lactic acid bacteria (LAB) are known to be able to produce antihypertensive bioactive peptides in fermented foods. Angiotensin converting enzyme (ACE) plays a fundamental role in the *Renin-Angiotensin System*, which can increase blood pressure through the inactivation of decapeptide conversion process of Angiotensin I into Angiotensin II. ACE is one of the effective targets for reducing hypertension. ACE inhibitory (ACE-I) peptides had the ability to inhibit ACE by binding to the active site of the ACE enzyme. ACE-I activity were associated with the presence of aromatic and aliphatic amino acids such as Pro, Phe and Tyr on C-terminal and Val and Ile at N-terminal. The formation of ACE-I peptides in fermented foods is associated with proteolytic activity of LAB during fermentation. LAB is able to secrete extracellular proteinases breaking down proteins into simpler molecules. ACE-I peptides are generally short peptides or tripeptide consist of 2 to 20 amino acid residues with a molecular weight range of <5 kDa. The formation of ACE-I peptides in fermented foods is influenced by the LAB strain, substrate and fermentation condition. This review aimed to provide information related to formation of ACE-I peptides by lactic acid bacteria in fermented foods, the mechanism of and the factors influence the formation of ACE-I peptides.

1.Introduction

Lactic Acid Bacteria (LAB**)** are a large group of microorganisms naturally found in the gastrointestinal and urogenital tracts of humans, animals, and various fermented as well as non-fermented foods. The groups of LAB include *Lactobacillus, Streptococcus, Lactococcus, Pediococcus, Streptococcus, Leuconostoc, Oenococcus, Carnobacterium, Weisella and Tetragenococcus,* with the main characteristic of Gram-positive, round or rod, non-spore forming, capable of fermenting carbohydrates and catalase negative. LAB is characterized as facultative anaerobic or microaerophilic and anaerobic bacteria (Axelsson, 2004). LAB has been known to have functional properties conferring health beneficial effect including shortening of diarrhea duration, protecting against enteropathogenic bacterial infections, necrotizing enterocolitis (NEC) and inflammation of the stomach (Culligan *et al*., 2009; Vasiljevic and Shah, 2008), improving

lactose metabolism, decreasing cholesterol, decreasing risk of mutagenicity and carcinogenic and also stimulate the immune system (Kimoto-nira et al., 2007; Lee et al., 2011; Saad et al., 2013).

LAB isolated from breast milk had been demonstrated to be able preventing diarrhea (*L. rhamnosus* strain R23) (Nuraida et al., 2012), and assimilating cholesterol (*Pediococccus pentasaceus)* (Nuraida et al., 2011). One of the functional LAB properties which quite interesting it was their potential to produce antihypertensive peptides in various fermented foods. Antihypertensive peptides had the ability to inhibit Angiotensin Converting Enzyme (ACE).

ACE: peptidyldipeptide hydrolase, EC 3.4.15.1 were metals containing Zinc, located in the endothelial layer of the blood vessels in the lungs that plays an important role in regulating blood pressure (Jung et al., 2006). ACE increases blood pressure throught process of inactivating decapeptide Angiotensin I into Angiotensin II as an active form. The conversion process is carried out through the release of dipeptides at C-terminals from angiotensin I to form angiotensin II is a potent vasoconstrictor (Riordan, 2003), that being able to cause an increase in blood pressure or a very hypertensive compound. ACE will hydrolyze vasoactive bradykinin (Fitzgerald, 2006), stimulate an increase in aldosterone secretion in the adrenal cortex (Cheung et al., 1980) so that it causes vasoconstriction and fluid retention which is one of the causes of hypertension. Efforts to decrease blood pressure in patients with hypertension include non-pharmacologically through lifestyle changes and pharmacologically by administering antihypertensive drugs. Synthetic drugs such as captopryl, ala cepryl, and lisinopryl were widely used for the treatment of hypertensive patients, however the side effects that arise were symptoms of hypersensitivity in the form of hives and symptoms of upper respiratory tract infections such as coughing**.** Antihypertensive peptides in fermented foods produced by LAB is one of the natural sources that potentially used in prevention or treatment of hypertension. The peptides can be isolated from fermented foods, such as fermented fish, pear juice, milk and dairy products. Several researchers had succeeded isolating LAB from fermented foods that have the potential to produce angiotensin converting enzyme inhibitory (ACE-I) peptides. Among different LAB, *Lactobacillus helveticus* has often been used widely as a starter culture in dairy products to produce ACE-I peptides (Chen et al., 2015; Wang et al., 2015). These species has a Generally Recognized as Safe (GRAS) status and reported also has a pharmacological target which promises to reduce blood pressure. Calpis is Japanese-made soft drink made from skim fermented by *L. helveticus* CP 790 and *Saccharomyces cerevisiae* (Nakamura et al., 1995b). Calpis had two ACE-I dipeptides Val-Pro-Pro (VPP) and Ile-Pro-Pro (IPP) that were similar structure as captopril and enalapril (De Castro and Sato, 2015) which are commonly used for the treatment of hypertension. The purpose of this paper was to provide information on the role of LAB in the formation of ACE-I peptides in fermented foods, the mechanism of its formation, and factors influencing ACE-I peptides formation in fermented food.

2. ACE-I Bioactive Peptides

Bioactive peptide were defined as specific part or fragments of proteins that have a positive impact on body function, which can affect overall health status. The protein fragments have biological activities such as antioxidants, antimicrobial,antithrombotic, antiinflammatory, and antihypertensive (Korhonen, 2009; Choi et al., 2012; de Castro and Sato, 2015; Sanjukta and Rai, 2016). The functional activities of these bioactive peptides were based on the composition and sequence of amino acids. There was a relationship between the size and structure of amino acid peptides and ACE-I activity. ACE-I peptides were generally short peptides or tripeptides with 2-5 amino acid residues with molecular weight ranges <5 kDa (Minervini et al., 2003; Lignitto et al., 2010). Several other studies state that there were 2-20 amino acid

residues (Möller et al., 2008; Phelan and Kerins, 2011; Norris and FitzGerald, 2013)**.** Research conducted by Yamamoto et al. (1994) stated that *Lactobacillus helveticus* CP790 could produce 25 types of short peptides in milk that have ACE-I activity. In traditional fermented fish called "bekasam" Wikandari and Yuanita, (2016) found a short peptide type having ACE-I activity and being resistant to gastrointestinal proteases (pepsin and trypsin). Angiotensin-1-converting enzyme in humans consist of two form;(1) Somatic (sACE) and, (2) germinal/testicular (gACE). The form both encoded by the same gene lacated on cromosome 17 at q23, sACE is a type-I membrane bound protein that consist of a-28 residue C- terminal cytosolic domain, a 22-residue hydrophobic trans membrane domain and 1227-residue extracellular domain that is heavily glycosylated and further divided into a 612- residue N- terminal domain, linked by a 15 residue sequence to a 600-residue C-terminal domain (Zisman, 1998; Riordan, 2003)

The C-terminal domain of is primarily involved in blood pressure regulation, while the N-terminal domain is involved in control of hematopoietic stem cell differentiation and proliferation. Commercial antihypertensive drugs such as Captopril, Lisinopril, and Enalapril had similar mechanism in interacting with the active site of ACE with the domains of C and N, both on sACE or gACE (Riordan, 2003; Sturrocka et al., 2004). Both C- and N-domains containing an active site the sequences His-Glu-XX-His which serves as the zinc binding ligand. These active sites are located within the cleft of the two domain, and are protected by an N-terminal ‘ lid’. This ‘lid’ block access of large polypeptide to the active site. This is thought to explain why small peptide are more effective in inhibition ACE (Gobbetti et al., 2002; Fandiño et al., 2006). The QSAR (Quantitative structure-activity relationship modelling)show that the C-terminal of the peptide had principal importance on ACE inhibitory activity, with hydrophobic C-terminal residue being essential for high potency (Wu et al., 2006b; Wu et al., 2006a)

The active site of ACE has three sub-sites, including S1 (antepenultimate), S1′ (penultimate) and S2 (ultimate) which have different characters to bind three C- terminal amino acids substrates or inhibitors located on two homologous active sites (Brew, 2003). Competitive substrates or inhibitors containing hydrophobic amino acid in C-terminal position were preferred by ACE. To enable interaction between enzymes and inhibitors, the substrate (the inhibitors) must be bound to three sub-sites of the active site of the enzyme with different amino acid sequences (Escudero et al., 2010). Valine-Proline-Proline (Val-Pro-Pro) and Isoleucine-Proline-Proline (Ile-Pro-Pro) were tripeptide produced by *Lactobacillus helveticus* (Nakamura et al., 1995) one of the two well-known antihypertensive peptides had highest ACE-I activity had the same C-terminal sequence. Pro residue at ultimate C-terminal, explaining their high ACE-I activity.

ACE-I activity were associated with the presence of aromatic and aliphatic amino acids such as Pro, Phe and Tyr on C-terminal and Val and Ile at N-terminal (Fuchs et al., 2008; Wijesekara et al., 2011; Jao et al., 2012). Various aromatic AA of ACE-I peptides with in molecular size and activity is presented in Table 1. Hydrophobic amino acids such as Try, Phe, Ala, Ile, Val and Met or positively charged amino acid such as Arg, Lys and Pro at the terminal position peptide show an association with ACE-I activity (Rai et al., 2017). Rodríguez-Figueroa et al. (2012) showed that HPHPHLSFMAIPP peptide fraction had hydrophobic amino acids (Pro) and DDQNPH peptides with histidine residues at C-terminal responsible for high ACE-I activity in fermented milk using *L. lactis* NRRL B-50571.

Daliri et al. (2018) stated that PFNL and FNL peptides had the highest ACE-I activity (IC_{50}: 0.048 and 0.038 mg / mL) of 8 ACE-I peptide found in fermented soy milk using *L. casei* spp. *pseudoplantarum*. The FNL peptide has hydrophobic amino acids (Proline and Phenylalanine) in N-terminal and Leucine in C-terminal.

Bioactive peptides of ACE-I have been classified into 3 groups: (1) true inhibitor type, (2) substrate type, and (3) pro-drug type. IC_{50} values of the true inhibitor is not altered by preincubation with ACE. The substrat type is altered by preincubation with ACE and pro-drug type being converted to true inhibitor type by ACE or gastrointestinal proteases (Fujita et al., 2000). The study Fujita & Yoshikawa, (1999) reported the peptides Leu-Lys-Pro-Asn-Met (IC_{50}:2.4 μM) was hydrolyzed by ACE and 8-fold increased to produce Leu-Lys-Pro (IC_{50}: 0.32 μM)

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Table 1. **Aromatic AA of ACE-I peptides in MW produced by various LAB in fermented milk**

Aromatic of AA ACE-I peptides	Molecular Weight	ACE-I activity (%) / IC_{50}	LAB producer	References
LI AKAA	<1 kDa	68.21 ± 1.06	*L. casei*	Li et al. (2017)
LHLPLP VRGPFPIIV VLGPVRGPFP	<3 kDa	IC_{50}: < 100 μg protein/mL	*E. faecalis* BCS27	Gútiez et al. (2013)
LQSW PEQSLVYP MFPPQSVLSLSQS LLYQEPVLGP KPAAVRSPAQILQWQV IHAQQK	<3 kDa	IC_{50}: 5 ± 2 μg /mL	*L. plantarum* PU11 and *Lb. lactis* DIBCA2	Nejati et al. (2013)
YQDPRLGPTGELDPA TQPIVAVHNPVIV, PKDLREN LLLAHLL NHRNRMMDHVH	<3 kDa	IC_{50} : 43.52 ± 0.61 mg/L IC_{50} : 7.78 ± 0.29 mg/L IC_{50} : 4.52 ± 0.15 mg/L IC_{50} : 19.60 ± 0.25 mg/L	*Koumiss cultures*	Chen et al. (2010)
LVYPFPG,PIHNSLPQN LVYPFPGPIH	<3 kDa	IC_{50}: 71 μM IC_{50}: 89 μM	*L. jensenii*	Pihlanto et al. (2010)
LVYPFP LPLP	≤3 kDa	IC_{50} : 132 μM IC_{50} :703 μM	*Bifidobacterium bifidum* MF 20/5	Gonzalez-Gonzalez et al.(2013)
QEPVLGPVRGPFPIIV YPSYGL HPHPHLSFMAIPP SLPQNIPPL	<3 kDa	0.041 ± 0.003 μg/mL 0.034 ± 0.002 μg/mL	*Lc. lactis* NRRL B-50572 *Lc. lactis* RRL B-50571	Rodríguez-Figueroa et al. (2012)
LHLPLP LVYPFPGPIPNSLPQNIPP	≤ 3 kDa	IC_{50} : ≤ 5 μM	*E. faecalis* CECT 5727	Quirós et al. (2007)
LVESPPELNTVQ VLESPPELN WGYLAYGLD	≤ 10-kDa	IC_{50}: 0.11 μM IC_{50}: 0.23 μM IC_{50}: 0.10 μM IC_{50}: 0.03 μM IC_{50}: 0.03 μM	*L. casei* and *kombucha cultures*	Elkhtab et al. (2017)

Aromatic of AA ACE-I peptides	Molecular Weight	ACE-I activity (%) / IC_{50}	LAB producer	References
VAPFPEVFGK LVYPFPGPLH FVAPEPFVFGKEK		IC_{50}: 0.75 µM		
DKIHPFAQ,TQTPVVVP, KAVPQ, RPKHPIKH	≤ 3 kDa	IC_{50}: 39 µM - 257 µM	*L. delbrueckii S. thermophilus* *L. paracasei*	Papadimitriou et al. (2007)
EDEVSFSP EVSFSP SFSP RSPFNL SRPFNL ENPFNL PFNL FNL	≤ 7kDa	IC_{50}: 0.571 ± 0.12 mg/mL IC_{50}: 0.133 ± 0.03 mg/mL IC_{50}: 0.262 ± 0.18 mg/mL IC_{50}: 0.811 ± 0.05 mg/mL IC_{50}: 0.131 ± 0.02 mg/mL IC_{50}: 0.287 ± 0.07 mg/mL IC_{50}: 0.048 mg/mL IC_{50}: 0.038 mg/mL	*Pediococcus acidilactici* SDL1414	Daliri et al. (2018)
AFPEHK	10 kDa	33.19 ± 2.768 37.77 ± 10.222	*L. casei* (NK9) *L. fermentum*	Parmar et al. (2017)
LIVTQ LIVT	10 kDa	IC_{50}: 0.087 µM IC_{50}: 0.110 µM	*L. casei* spp. *pseudoplantarum*	Vallabha and Tiku, (2013)
RPKHPIKHQGLPQEVEV LNENLRF FVAPFPEVFGK YQEPVLGPVRGPF YQEPVLGPVRGPFPI YQEPVLGPVRGPFPIIV	<3 kDa	IC_{50}:<10 µg/mL	*Lc. lactis* ssp/ mix culture *lactis–E.faecium*	Torres-Llanez et al. (2011)

3. LAB of fermented foods producing ACE-I peptides.

Fermented foods have been known and consumed for a long time. Fermentation involves microorganisms that can take place spontaneously or by using a culture starter. Lactic acid bacteria are known as bacteria that are involved in many fermented foods such as fermented milk, meat, legumes and vegetables. A fermented milk product are classified into two major groups on the basic on of microorganism: (1) lactic fermentation, (2) lactic-fungal fermentations (Mayo et al., 2010).

ACE-I activity were found in fermented dairy products such as cheeses (Lignitto et al., 2010; Qureshi et al., 2013; Lu et al., 2016), *dahi* - Indian yogurt (Ashar and Chand, 2004), caprina kefir (Quirós et al., 2005), koumiss (Chen et al., 2010), sheep milk yoghurt (Papadimitriou et al.,

2007), fermented camel milk (Moslehishad et al., 2013), fermented goats milk (Minervini et al., 2009). ACE-I activity was also found in *douchi* (Zhang et al., 2006), fermented fish (bekasam, hezhiko, narezushi), fermented oyster and fermented pear juice (Itou et al., 2007; Ankolekar et al., 2012; Wikandari et al., 2011; Wenno et al., 2016).

The occurrence of ACE-I activity in fermented foods has encouraged many researchers to isolate LAB present in fermented foods. Various LAB producing ACE-I peptides from different fermented food is presented in Table 2. Barla et al. (2016) isolated 52 LAB isolates from various traditional Japanese fermented foods (*Kaburazushi, Narezushi, Konkazuke,* and *Ishiru).* Isolate of *Lactobacillus brevis*, *Lactobacillus buchneri* and *Weissella hellenica* that used as starter cultures in soya and milk fermented were show to posses ACE-I activities (IC_{50}:<1mg protein/mL). Similarly, Wikandari et al. (2011) have isolated 150 LAB from *bekasam* (fermented fish). ACE-I activity of *bekasam* was 51.77%-65.75%. Li et al. (2017) reported that among 41 strains of *L. casei* isolated from fermented food of Tibet, Mongolia, Sichuan and Gansu, 22 strains used as culture starter in fermented milk and showed ACE-I activity above 60%. Chen et al. (2015) has isolated 38 LAB groups of *L. helveticus* from traditional fermented milk and revealed that fermented milk produced with 3 strains of *L. helveticus* (IMAU80851, IMAU80852 and IMAU80872) showed ACE-I activity of 75%. Other LAB groups such as *Lactococcus* and probiotic bacteria that used as starters to make cheddar cheese were capable of producing ACE-I peptides (Ong and Shah, 2008). *Leuconostoc* spp, *S. thermophilus*, *Lactococcuc lactis*, *L. helveticus* and *L. delbrueckii* have also been reported to be able producing ACE-I peptides (Kilpi et al., 2007; Gútiez et al., 2013). LAB group *Lactobacillus* and *Lactococcus* were generally used as starter cultures. *Lactobacillus* was known to have both high proteolytic and ACE-I activities, while *Lactococcus* has ability to degradate lactose in milk and produce ACE-I peptides (Kuipers, 2001; Rodríguez-Figueroa et al., 2010).

In vitro screening of lactic acid bacteria for producing ACE-I peptides could be done with two approaches: (A) the enzymatic characteristics of the bacterial proteinases and, (B) The ability of strain to reduce ACE activity (Beltrán-Barrientos et al., 2016). *Lactobacillus helveticus* species, produces abundant intracellular enzymes, including cell-envelope proteinase, endopeptidases, aminopeptidases, and the X-prolyl dipeptidyl aminopeptidase, PepX (Exterkate, 1995).

4. Factors affecting formation of ACE-I peptides in fermented foods

The formation of ACE-I bioactive peptides in fermented foods influenced by several factors. The main factors that are widely observed and reported by researchers include the type of LAB (strain of starter culture), inoculum density, fermentation time, and substrate composition **(**Li et al., 2017; Shi et al., 2016)

4.1. Starter Culture

ACE-I activity produced in a fermentation foods is largely determined by starter culture. Some types of LAB which are isolated from fermented and non-fermented foods produce ACE-I inhibitory with varies activity (Gobbetti et al., 2000; Gútiez et al., 2013; Rodríguez-Figueroa et al., 2010; Kilpi et al., 2007). During the fermentation process, milk protein was hydrolyzed to produce a bioactive peptides by proteolytic enzymes which produced by starter culture. The activity, size and sequences of ACE-I peptides formed are strongly influenced by the type of LAB starter culture. *L. helveticus* is reported to produce ACE inhibitors peptide namely IPP and VPP in various milk product (Pan & Guo, 2010; Yamamoto et al., 1994; Tsai et al., 2008; Chen et al., 2010) with ACE-I activity 9-74.5% (Wang et al., 2015). Other LAB that are known to be able to produce ACE-I activity are *L. jensenii* (Pihlanto et al., 2010), *Lactococcus lactis* (Kuipers, 2001), and *Enterococcus* (Quirós et al., 2007; Hati et al.,

2015). Research by Chen et al. (2015) showed that ACE-I activity of *Lactobacillus* with different activities, i.e. *L. reuteri* of 95.92%, *L. bulgaricus* of 84.61%, *L. rhamnosus* of 82.79% and *L. helveticus* of 78.57%.

Table 2. ACE-I activity of various LAB in different fermented foods

Lactic Acid Bacteria	Fermented foods	ACE-I activity (%) /IC_{50}	References
L. plantarum 417	yogurt of goat milk	90.70 ± 1.27%	Sathya et al. (2017)
L. delbrueckii subsp. *bulgaricus* ACA-DC 87 *S. thermophilus* ACA-DC 835	yogurt of sheep milk	75% 92%	Georgalaki et al. (2017)
L reuteri, *L. bulgaricus,* *L.rhamnosus* *L. helveticus*	yogurt of goat milk	95.92% 84.61% 82.79% 78.57%	Chen et al. (2015)
S. thermophilus CR12 *L. casei* LC01 *L. helveticus* PR4	yogurt of goat milk	82.0%	Minervini et al. (2009)
L. plantarum C2	soybean milk	75.97 ± 1.5 %	Singh and Vij, (2017)
L. casei	soybean milk	IC_{50}: 0.89-4.31mg mL	Bao and Chi, (2016)
L. buchneri *Weisella hellenica*	soybean milk	IC_{50} : 1.33 ± 0.04 mg/mL^{-1} IC_{50} : 1.37 ± 0.02 mg/mL^{-1}	Barla et al. (2016)
L. plantarum B1765	*bekasam* (fish fermented)	68.17 ± 1.32%.	Wikandari et al. (2012)
L. acidophilus	pear juice	≤ 50 %	Ankolekar et al. (2012)
Lactococcus lactis ssp. *lactis* *Enterococcus faecium*	cheese mexican fresco	IC_{50} : 5.2 ± 0.10 µg mL IC_{50}:10.4 ± 0.40 µg mL	Torres-Llanez et al. (2011)
L. helveticus 881315	yogurt of bovine milk	IC_{50}: 16.91 ± 0.25 mg mL^{-1}	Shi et al. (2016)
L. delbrueckii ssp. *bulgaricus* LB340	yogurt of bovine milk	67.71 ± 7.62 mg mL^{-1}	Qian et al. (2011)
L. rhamnosus PTCC 1637	yogurt of camel milk	IC_{50}: 1.45 ± 0.01 mg mL^{-1}	Moslehishad et al. (2013)
L. rhamnosus NS4	yogurt of camel milk	79.66%	Solanki and Hati, (2018)
L. casei	yogurt of bovine milk	68.21 ± 1.06%	Li et al. (2017)

Lactic Acid Bacteria	Fermented foods	ACE-I activity (%) /IC_{50}	References
L. helveticus H9	cow, mare and soybean milk	70.9-74.5%	Wang et al. (2015)
L. casei Shirota and *S. thermophiles*	yogurt of bovine milk (casein)	IC_{50}: 0.14 μg mL^{-1}	Rojas-Ronquillo et al. (2012)
L. helveticus LB10	yogurt of bovine milk	75.46%	Pan and Guo, (2010)
*L. helveticus (*H521, 4/149, 4/135, Hv25) *L. casei* 2465 *L. asidophilus*	yogurt of bovine milk	60-62% 66% 70%	Stefanova et al. (2009)
L. helveticus ND01	yogurt of bovine milk	69.51 ± 2.32 %	Sun et al. (2009)
Lactococcus lactis DIBCA2	yogurt of bovine milk	IC_{50} : 0.22 ± 0.03 mg/mL^{-1}	Nejati et al. (2013)
Bifidobacterium longum	yogurt of bovine milk	66.30 ± 2.43%	Ramchandran and Shah, (2008)
Bifidobacterium bifidum MF 20/5	yogurt of bovine milk	IC_{50}: 132 μM	Gonzalez-Gonzalez et al. (2013)
E. faecalis QA53	yogurt of bovine milk	IC_{50} : 24.3 μg /mL.	Gútiez et al. (2013)
E. faecalis CECT 5727	yogurt of bovine milk	IC_{50}: 28 ± 2 μg mL	Quirós et al. (2005)
E. faecalis	yogurt of bovine milk	IC_{50} : 34-59 μg mL	Muguerza et al. (2006)

4.2.Substrate

Substrates for fermentation affect ACE-I peptides formation by LAB. Wang et al. (2015) conducted a research using 3 types of milks to determine ACE-I inhibitory activity by *L. helveticus* H9. The milks are nonfat cow milk powder, mare milk powder, and nonfat soy milk powder. The results of the study showed that ACE-I activity was found in all three types of milks (above 50%). However, the highest ACE-I activity was found in cow milk (65.1-77.2%), followed by horse milk (61.7%) and soy milk (42.1- 64.9%). The results also showed that two types of tripeptide, VPP and IPP were only found in cow milk not in soy milk or mare milk. The two types of tripeptide were previously have been shown as ACE-I peptides produced by *L. helveticus* (Stefanova et al., 2009; Chen et al., 2014). *L. helveticus* H9 takes a shorter time to produce ACE

inhibitors in cow's milk than horse milk and soy milk. Casein was the largest component in cow's milk (80%) and whey protein (20%). Protein content in horse milk was lower than in cow's milk. ACE-I peptides are formed by bacterial proteinases when milk protein particularly casein, are hydrolyzed into oligopeptides as nitrogen source for bacterial growth.

Bioactive peptides have been derived from casein (αS1-CN, β-CN, κ-CN) (Torres-Llanez et al., 2011; Lu et al., 2016). Fragment of β-CN, 84–86 [β-CN (f84–86) were found in AA sequence of VPP and fragment β-CN(f74–76) and κ-CN(f108–110) in AA sequence of IPP. Soybeans contain high stachyose and raffinose which are limiting factors for LAB growth and to produce ACE-I peptides. However, some

researchers showed ACE-I activity in fermented soy milk with IC_{50}: 0.28-4.34 mg/mL (Donkor et al., 2005; Vallabha and Tiku, 2014; Bao and Chi, 2016). Major component of soybean protein is glycinin (11S globulin) and β-conglycinin (7S globulin) accounted to approximately 40% and 30%, respectively of the total protein (Utsumi et al., 2002). Study by Gibbs et al. (2004) showed that glycinin was the precursor of 95% of the peptides formed by soybean protein hydrolysis. Nowadays milk from various sources (cows, buffaloes, goats, sheep, camels and yaks) has been widely used to produce ACE-I (Ao et al., 2012; Fadda et al., 2010; Papadimitriou et al., 2007; Moslehishad et al.,2013)

Protein in fermented foods are the natural source of antihypertensive peptides. The protein content in milk was divided into two categories: insoluble protein (casein group) and dissolved protein (whey protein), generally found in lactoserum. The casein group consists of several types namely αs1-, αs2-, β-, K- and γ-casein, while whey protein consists of beta-lactoglobulin, alpha-lactalbumin, lactoferrin, immunoglobulin, serum albumin, glycomacro-peptides, enzymes and growth factors. ACE-I activity in fermented cow's milk was reported to reach ≥ 50%, whereas in goat's milk ACE-I activity was 60-85% (Quirós et al., 2005; Minervini et al., 2009). Goat's milk has α-casein content that lower than cow;s milk, and in contrast β-casein was the majority protein content in goat's milk (Jandal, 1996)

4.3. Inoculum Density

The inoculum density of LAB is reported to influence the ability of LAB to produce peptides with ACE-I activity (Wang et al., 2015; Shu et al., 2015; Li et al., 2017). A study to determine the effect of inoculum density on ACE-I activity in cow's milk. The density *L. helveticus* as inoculum of 1×10^6 CFU /mL produced the highest ACE-I activity of 74.97% compared to three other inoculum densities that are 5×10^6 , 1×10^7, and 5×10^7 CFU/mL (Chen et al., 2015). Similar result were reported by Li et al. (2017) using *Lactobacillus casei* which showed that the inoculum density of $1x10^6$ CFU/mL produced higher ACE-I activity (73.50%) in cow's milk than two other inoculum densities of 5×10^6 and 1×10^7 CFU/mL. Wang et al. (2015) using 4 different *L. helvetikus* H9 inoculum densities, i.e. 2×10^6, 5×10^6, 1×0^7, and 2×0^7 CFU/mL also showed that the use of the 5×10^6 inoculum density resulted in the highest ACE-I activity (70.9-74.5%). The density of the inoculum also seems to be influenced by the type of LAB.

The use of high inoculum density does not always correlate with the high ACE-I activity. It is assumed that the use of a high inoculum density causes an increase the growth rate of LAB resulting in rapid accumulation of acids in the medium as cells metabolite. The acid can suppress proteolytic activity to degrade proteins to produce peptides that have the potential as ACE-I peptides. Previous research by (Samona et al. (1996) and Wang et al. (2015) explained that the density of the inoculum used would determine the rate of increase in total acid in the milk fermented by *L. acidophilus* and *Bifidobacterium.*

4.4. Fermentation Time

Each of LAB has the optimum temperature and time range for its growth and for peptides production. Different temperature and time of fermentation were used for ACE-I peptide formation, i.e 37 ^{0}C for 24 hours used by Fuglsang et al. (2003); Moslehishad et al. (2012); Chen et al. (2014) and Li et al. (2017) and temperatures of 30 ^{0}C for 48 hours used by Muguerza et al. (2006); Quiros et al. (2007); Rodríguez-Figueroa et al. (2012). Li et al. (2017) found that incubation at 30 ^{o}C significantly increrased ACE-I activity in fermented milk by *L. casei* compared to two other temperatures i.e. 33 and 40 ^{o}C with the maximum ACE-I activity of 84.84 ±1.23%. Otte et al. (2011) also found that incubation temperature of 37 °C was optimum for the production peptides with ACE-I activity in milk fermented by *Lactococcus lactis.* Another study evaluated the effect of temperature and incubation time of ACE-I activity in milk by *Lactobacillus plantarum* LP69 (Shu et al.,

2015). The temperatures used are 25, 30,35, 40 and 45 ^{0}C.

The incubation time was 0 to 36 hours. The optimum ACE-I activity was obtained at an incubation temperature of 35 °C for 14 hours with ACE-I activity of 81.25%. It is assumed that ACE-I activity increased with the increase of LAB counts. This incubation time was slightly different from previous studies on milk fermented by *Lb. bulgaricus* LB6 (Shu et al., 2015) where the highest ACE-I activity was obtained at 12 hours incubation time.

Pihlanto et al. (2010) also found that the optimum ACE-I activity of milk fermented by *L. acidophilus* ATCC 4356 and *L. jensenii* ATCC 25258 were obtained after 20 hours incubation. Similar study results showed by Gonzalez-Gonzalez et al. (2013) reported that the incubation time required by *Bifidobacterium bifidum* MF 20/5, *L. salivarius* NCIMB 11975, *L. reuteri* NCIMB 11951, *L. casei* YIT 9029, and *L. plantarum* NCIMB for optimum was 24 hours with ACE-I activity above 85%. Those results show that each strain had a different optimum temperature and incubation time to produce peptides with high ACE-I activity. However, Chen et al. (2015) reported that the incubation temperature did not have a significant effect on ACE-I activity. The incubation temperature of 33, 37, and 40 °C did not affect significantly to ACE-I activity in milk fermented by *L. helveticus* IMAU80872.

5. Conclusions and future perspective

Lactic acid bacteria with proteolytic activity have the potential to produce ACE-I bioactive peptides. ACE-I bioactive peptides generally short peptides or tripeptides with a molecular weight of <5 kDa. The formation of bioactive peptides of ACE-I in fermented foods by LAB provides an opportunity to find new LAB strains with the ability to produce bioactive peptides of ACE-I. The selection of specific starter culture is crucial to deliver specific health properties. Substrate and fermentation conditions are other factors that can play important roles for the formation of bioactive peptides of ACE-I. Potential LAB strains that produce bioactive peptides of ACE-I can be applied as starter cultures or as a food component to develop functional food that recently becoming popular for maintaining human health. In vivo studies also confirm that foods fermented by certain strains of LAB are potential to be used as antihypertensive. This open an opportunity to obtain natural antihypertensive from foods that can be used in the treatment or prevention of hypertension.

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