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RESEARCH ARTICLE

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Insight into the bovine milk peptide LPcin-YK3 selection in the proteolytic system of *Lactobacillus* species

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Antimicrobial peptides are class of small, positively charged peptides known for their broad-spectrum antimicrobial activity. Antimicrobial activities for most antimicrobial peptides have largely remained elusive, particularly in the lactic acid bacteria. However, recently our investigation using LPcin-YK3, an antimicrobial peptide from bovine milk, suggests that in vitro antimicrobial activity was reduced over 100-fold compared with pathogenic bacteria. Additionally, for the structural study of how antimicrobial peptide undergoes its reaction at the proteolytic pathway of lactic acid bacteria based on degradation assay and propidium iodide staining, we performed molecular docking for interaction between oligopeptide-binding protein A and LPcin-YK3 peptide. Given that degradation related to the LPcin-YK3 peptide in lactic acid bacteria proteolytic system, the inhibitory inactivity of LPcin-YK3 against beneficial lactic acid bacteria strains may be one of the primary pharmacological properties of recombinant peptide discovered in bovine milk. These results provide structural and functional insights into the proteolytic mechanism and possibility as a putative substrate of oligopeptide-binding protein A in respect of LPcin-YK3 peptide.

KEYWORDS
antimicrobial peptide, cell envelope proteinase, lactic acid bacteria, oligopeptide permease, proteolytic system

1 | INTRODUCTION

Lactic acid bacteria (LAB) are defined as Gram-positive and facultative anaerobic bacteria with a fermentative metabolism. LAB are characterized by their production of lactic acid and are predominant participants in much industrial and meat, vegetable products and dairy fermentations. Furthermore, LAB are indigenous habitants of the human gastrointestinal tract and are thought to be among the dominant colonists of the small intestine.[1] In particular, the fermented dairy products enriched with probiotic bacteria, that is, *Lactobacillus* and *Bifidobacterium*, have developed into one of the most successful categories of functional foods.[2,3]

From an industrial relevance point of view, a great deal of effort was made to investigate and manipulate the role of LAB as starter strains in the manufacturing processes of fermented foods. These LAB including *Lactobacillus* species are auxotrophic for numerous amino acids. An external source of amino acids or peptides is required for their growth, particularly in milk where concentrations of amino acids are low. To fulfill their amino acids requirement, *Lactobacillus* species have developed a proteolytic system, which hydrolyzes the

proteins and supplies the amino acids.[4] In general, the exploitation of casein by *Lactococcus* is initiated by a cell-envelope proteinase (CEP) that degrades the protein into oligopeptides. It is subsequently taken up by the cells via specific peptide transport systems for further degradation into shorter peptides and amino acids by a concerted action of various intracellular peptidases (Figure 1).[5–12] Although many LAB strains contain CEP, several of these strains do not, thus, they rely on starter LAB for the production of peptides and amino acids.

Given that presence of many putative starter LAB in the bacterial genome sequences and their diverse substrate specificities, the structural components of the proteolytic systems of LAB can be divided into three groups on the basis of their function: (i) proteinases that breakdown caseins to peptides, (ii) peptidases that degrade peptides, and (iii) transport systems that translocate the breakdown products across the cytoplasmic membrane.[6] Based on in vitro studies of the enzymes and transporters, a model for the comparable proteolytic pathway is proposed.[13–15] At present, three peptide transport systems have been characterized for LAB. The oligopeptide transport system (Opp) mediates the ATP-driven transport of peptides with four to at least eight residues. It plays a central role in the proteolytic pathway of LAB, as it is essential for the accumulation of all β-casein-derived amino acids. The dipeptide and tripeptide carrier of LAB (DtpT) is a unique transport system in contrast to the binding protein-dependent ATP-driven peptide transport systems. The DtpT system has a broad substrate specificity, but size recognition is restricted to dipeptide and tripeptide only. A third peptide transport system (DtpP), the specificity of DtpP partially overlaps that of DtpT. DtpP transports preferentially dipeptide and tripeptide that are composed of hydrophobic (branched-chain amino acid) residues, whereas DtpT has a higher specificity for more hydrophilic and charged peptides. Moreover, its main features are (i) proteinases have broad specificity and are capable of releasing a large number of different oligopeptides, of which a large fraction falls in the range of four to eight amino acid residues; (ii) oligopeptide transport is the main route for nitrogen entry into the cell; (iii) all peptidases are located intracellularly and concerted action of peptidases is required for complete degradation of accumulated peptides. In studies that have previously been reported, the published genome sequences of starter LAB include *Lactococcus lactis*,[16] *Streptococcus thermophilus*[17] and *Lactobacillus sakei*.[18] This comparative genomics reveals some differences between the proteolytic systems of LAB, differences that are thought to reflect the various environmental niches these bacteria occupy.[19]

By this time, most attention was being paid to the proteolytic system of lactococci, which is by far the best-studied, and the second-best unraveled proteolytic systems are those of *Lactobacillus* species such as *Lactobacillus helveticus*, *Lactobacillus delbruckii* and *Lactobacillus casei*. Nevertheless, very little information is available on the transport of decomposition products related to the antimicrobial peptide (AMP) in these *Lactobacillus* proteolytic pathways. Recently, AMP derived from bovine milk (LPcin-YK3) has antimicrobial effects on *Candida albicans* as well as Gram-positive and Gram-negative bacteria in vitro,[20] although the possibility of using it as an antibiotic substitute still remains elusive. This study demonstrated that LPcin-YK3 could be good as therapeutic additives for skin diseases, and in the cosmetic industry, and furthermore suggested that it might help to prevent the growth of pathogenic bacteria and to improve skin health.

In the present research, we produced consisting of 15 amino acids based on often occurring amino acids in AMPs. Moreover, the 3D model of AMP was generated for this sequence by the PEP-FOLD 3 online tool.[21] Furthermore, we investigated in vitro tests of its antimicrobial effect, stability in heat and different pH solutions, degradation assay by *Lactobacillus* species and propidium iodide (PI) staining. Eventually, the structural and functional analyses of the structural components are closely related to the proteolytic system in LAB performed to study the action mechanism of the peptide. The structural information gained from this study

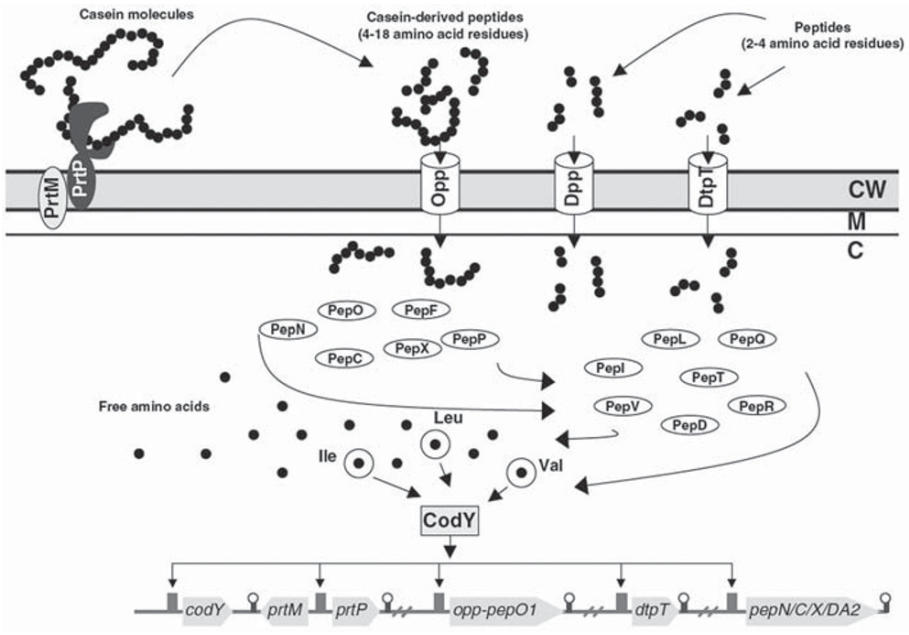

FIGURE 1 Schematic representation of the proteolytic system for casein proteolysis, transport, peptidolysis, and regulation (data from Savijoki et al[5]). CW, cell wall; M, membrane; C, cytoplasm; PrtP, cell-envelope proteinase; Opp, oligopeptide permease, DtpT, the ion-linked transporter for di- and tripeptides, and Dpp is an ABC transporter for peptides containing 2 to 9 amino acid residues. The transcriptional repressor CodY represses the expression of genes comprising the proteolytic system in *Lactococcus lactis*

would provide insights into how AMP undergoes its reaction toward the proteolytic system.

2 | MATERIALS AND METHODS

2.1 | Expression and purification of LPcin-YK3 peptide

LPcin-YK3 was purified as reported previously.[20] Briefly put, the recombinant protein containing a ketosteroid isomerase (KSI) fusion partner and His_6-tag to facilitate purification was expressed in a mutant *Escherichia coli* C41(DE3) strain (Novagen, Darmstadt, Germany). The cells were cultured in Luria-Bertani (LB) medium containing ampicillin and transferred to M9 minimal medium and induced with 1mM isopropyl β-D-1-thiogalactopyranoside (IPTG, Amresco, USA) addition.

2.2 | Characterization of LPcin-YK3 peptide

The LPcin-YK3 (purity >95%) was finally purified by reverse-phase high-pressure liquid chromatography (RP-HPLC, Waters Binary 1525, USA). The correct molecular mass of the purified LPcin-YK3 peptide was confirmed by Ultraflex III matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) equipment (Bruker Daltonics) (Figure 2). The 10 mg/mL of α-cyano-4-hydroxycinnamic acid (CHCA, Sigma, USA) matrix was used and dissolved in 30% acetonitrile and 0.1% trifluoroacetic acid (TFA). The LPcin-YK3 peptide was dissolved in the matrix solution, and this was loaded on the plate using the dried droplet method. The mass spectrum of LPcin-YK3 peptide was acquired after averaging 1000 laser shots in positive ion reflector mode with the mass range of 100–6000 dalton (Da). A helical wheel projection of LPcin-YK3 demonstrated spatial segregation of its hydrophobic and charged residues (Figure 3), indicating the amphipathic nature of the LPcin-YK3 α-helix. The sequence of the 15 amino acid peptide LPcin-YK3 (NKVKEWIKYLKSLFS) contains a number of basic lysine (K) residues.

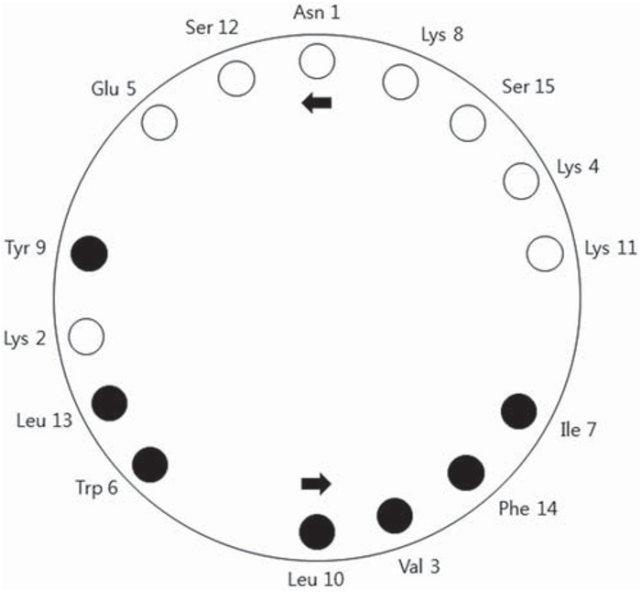

FIGURE 3 A helical wheel projection of LPcin-YK3 (15 amino acids). The incremental angle is 100°, the average hydrophobicity is 0.41 and the hydrophobic moment (μH) is 0.68. ●, hydrophobic residues; ◦, hydrophilic residues

Magainin[22] and many other AMPs also form amphipathic α-helices under conditions similar to those used in our experiments.

2.3 | Bacterial strains and growth media

Escherichia coli ATCC 12014, *Staphylococcus aureus* ATCC 25923, *Propionibacterium acnes* ATCC 6919, *Porphyromonas gingivalis* ATCC 33277 and *Streptococcus mutans* ATCC 25175 were grown in LB and Brain Heart Infusion (BHI) medium, respectively, whereas *Lactobacillus acidophilus* ATCC 4356, *Lactobacillus casei* ATCC 393, *Lactobacillus coryniformis* subsp. *coryniformis* ATCC 25602, *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830, *Lactobacillus fermentum* ATCC 14931, *Lactobacillus paracasei subsp. paracasei* ATCC 27216, *Lactobacillus plantarum subsp. plantarum* ATCC 10241 and *Lactobacillus rhamnosus* GG

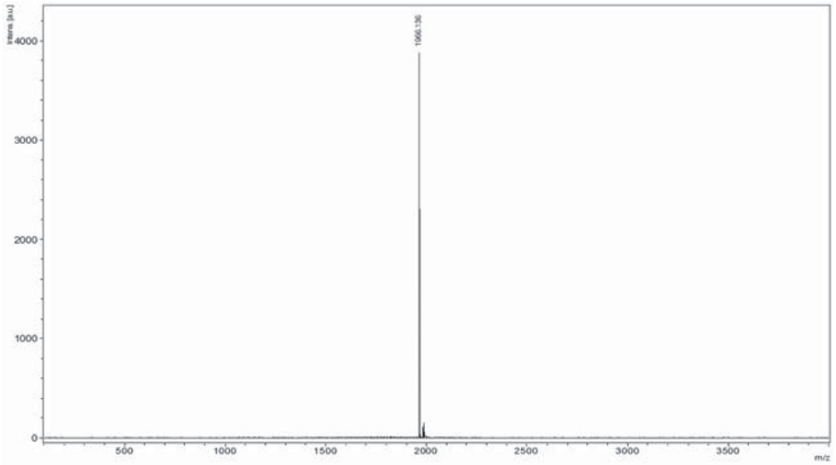

FIGURE 2 MALDI-TOF MS spectrometric data of the antimicrobial peptide LPcin-YK3. The measured molecular mass of monomeric LPcin-YK3 was 1966.13 Da, which agrees with the theoretical molecular weight of monomeric LPcin-YK3 (1966.32 Da) including the C-terminal homoserine lactone form

ATCC 53103 were grown in deMann, Rogosa and Sharpe medium (MRS; Becton-Dickinson, Sparks, MD). All LB, BHI and MRS broth cultures were incubated at 37°C and used to conduct the antimicrobial assay.

2.4 | Antimicrobial assay

The antimicrobial activity of LPcin-YK3 was tested by serial dilution titration method so as to specify the peptide minimum inhibitory concentration (MIC) against the different bacterial strains. Each bacterium was grown overnight at 37°C in the corresponding medium and was diluted in the same medium. Serial dilutions of the peptides were added to the microtubes in a volume of 200 μL, followed by the addition of 200 μL of bacteria to give a final inoculum of 5×10^4 colony-forming units (CFUs)/mL. The microtubes at different concentrations were incubated at 37°C for 24 h, after which time MICs were determined. The conventional antibiotic, ampicillin, and magainin-2 peptide were further used as a positive control. All tests were repeated three times independently, and the results were recorded as their mean ± standard deviation (SD).

2.5 | Stability of LPcin-YK3 peptide

The stability of LPcin-YK3 was determined upon heat treatment and in different pH solutions. LPcin-YK3 (10 μg/mL) was heated at 100°C for 60 min using a heating block (MK-10/20) and left for cooling at room temperature for 30 min. Following cooling, the antimicrobial activity of the peptide was determined against the *S. aureus* strain as described above. The pH stability of the antimicrobial activity (MIC) of the peptide was tested in LB at pH 3.7–9.4. The cell propagation in the presence or absence of peptide (0–5.0 μg/mL) was monitored by absorbance (OD_{600}) after 0 and 7 days at 22°C, respectively.

2.6 | Degradation assay of LPcin-YK3 peptide

The sensitivity of the peptide to putative cell-bound peptidase enzyme activity was tested by using resting cell suspensions of *S. aureus* and *Lactobacillus* strains prepared from the midexponential phase (OD_{600} = 0.5) cultures (20 mL/tube). The cultures were centrifuged at 10,000 g for 15 min at 4°C. Cell pellets were washed twice in 10 mmol/L phosphate buffer (pH 7.0) and reconstituted in 1 mL 10 mmol/L phosphate buffer, representing a 20-fold increase in cell density.[23] LPcin-YK3 (400 μg/mL) was added to the microtubes in a volume of 200 μL, followed by the addition of 200 μL of cell suspensions to give a final inoculum of 10^8 CFUs/mL. The reaction mixtures were incubated at 37°C for 30 and 60 min, after which time RP-HPLC was conducted. Furthermore, the resulting fragment peaks were analyzed *via* liquid chromatography-tandem mass spectrometer (LC-MS/MS, Agilent 6410B, RRLC system) at the Center for Research Facilities Kyung Hee University.

2.7 | Propidium iodide absorption assay

The PI staining method is based on the principle that PI cannot penetrate cell membranes of living cells, whereas cell membranes of dead cells permeate and combine with nuclei to show strong fluorescence. *S. aureus* and *Lactobacillus* strains were cultivated in LB and MRS medium up to the midlogarithmic phase, precipitated at 3000 rpm for 1 min. Cells were washed, and their number was adjusted to 10^5 CFUs/mL in phosphate buffer. The bacteria cells were incubated with 0.5, 1, and 2 times MIC (*S. aureus*) of LPcin-YK3 for 20 min at 37°C and used 0.5% Triton X-100 as a positive control. The cells were further washed in phosphate buffer and incubated with 1 μL of PI (5.0 μg/mL) at 37°C for 30 min in the dark. The excitation was performed at a wavelength of 536 nm, and the resulting fluorescence was measured at 623 nm using a fluorescence spectrophotometer (Eclipse, Nikon Corp., Japan). The bacteria cells treated by PI in the absence of peptide were used as a negative control.

2.8 | Molecular docking of LPcin-YK3 peptide

Automated prediction of protein-protein interactions and protein-small molecule interactions is one of the most challenging problems in structural biology. The final goal of docking analysis is to find the correct association of two interacting molecules given a structural representation for each molecule separately. Here, we used PatchDock,[24] a very efficient algorithm for protein-small ligand and protein-protein docking. The molecular docking was determined by the PatchDock method based on shape complementarity principles with a monomer of the oligopeptide-binding protein A (OppA, Protein Data Bank accession 3DRG [25]) from *L. lactis* as a receptor molecule. The best-ranked docking solution of the purified peptide in the active site of OppA was obtained according to the geometric scores and desolvation energy value.

2.9 | Statistical analysis

All MIC results are reported as mean ± SD. Statistical analysis for the comparison of MIC values of LPcin-YK3 was performed by a *t* test by SPSS Statistics software (SPSS Inc. Chicago, IL, USA). The *P* values of less than 0.5 were considered as statistically significant.

3 | RESULTS

3.1 | Structural characterization of LPcin-YK3 peptide

The purity and identity of the final peptide were confirmed by MALDI-TOF MS. The molecular weights of the homoserine lactone form of LPcin-YK3 peptide after cyanogen bromide cleavage were 1966.13 Da (Figure 2). The result indicated that the molecular weight

of LPcin-YK3 matched well with the theoretical molecular weight and the purification was successful without further contamination.

In the previous study, the circular dichroism (CD) spectra of LPcin-YK3 were measured in the absence and presence of various dodecylphosphocholine (DPC) concentrations for examining the secondary structure and the behavior of the peptide in a membrane environment. LPcin-YK3 appeared unstructured in an aqueous environment with a negative peak around 195 nm. In the presence of DPC micelles, a clear transition between random coil and α-helix was observed with two negative peaks at 208 and 222 nm and a slightly positive peak at 192 nm, and thus, we suggested that the peptide had a stable α-helical structure in the presence of DPC micelles (see figure 3 of Kim et al[20]).

An in silico prediction was also performed using the online tool PEP-FOLD3. The 3D model prediction of LPcin-YK3 with the PEP-FOLD3 online tool indicated the presence of a perfect helix ranging from Val-3 to Phe-14 residues of LPcin-YK3 (Figure 4A). It is interesting that LPcin-YK3 is an amphipathic peptide which has a similar ratio of hydrophobic (Val-3, Trp-6, Ile-7, Leu-10, Leu-13 and Phe-14) and hydrophilic residues (Lys-4, Glu-5, Lys-8, Lys-11, Ser-12 and Ser-15), and shows cationic properties that facilitate to make a hole in the bacterial membrane (Figure 4B).

3.2 | Minimum inhibitory concentration determination for bacterial species

In this study, the antimicrobial spectrum of the recombinant peptide, LPcin-YK3 was preferentially analyzed against five pathogenic bacterial strains, including three Gram-positive and two Gram-negative strains using the liquid growth inhibition assay. Additionally, we compared the results with the antimicrobial activity of commercially available AMP (magainin-2) and antibiotic (ampicillin). Antimicrobial activity of all pathogenic bacterial strains tested by LPcin-YK3 are very similar to the within the scope of a MIC of 1.25 or 0.62 μg/mL, respectively, and the conventional antibiotic, ampicillin showed relatively high antimicrobial activity (5.0–7.5 μg/mL) of four to 12 times on that of LPcin-YK3. Although, the magainin-2 peptide with +3 net charges and a length of 23 amino acids showed a moderate to weak activity against *S. aureus* (20.6 μg/mL) and *S. mutans* (62.4 μg/mL).

Of the eight selected LAB strains, the LPcin-YK3 exhibited humble microbicidal behavior and had similar antimicrobial activity to that of magainin-2. Unlike pathogenic bacterial strains, it should be noted that much of LAB strains appear to be resistant to LPcin-YK3, resulting in the MIC value of over 100 μg/mL, except for only an *L. acidophilus* strain. However, the ampicillin antibiotic exhibited strong MIC values (0.62 or 2.50 μg/mL) for all eight LAB strains, unlike the LPcin-YK3 peptide. Details of MIC are listed in Table 1. These results suggest that the LPcin-YK3 peptide has probably strong growth-inhibitory effects on that of pathogenic bacteria than the LAB cell wall which has a complex structure with defined functions. In this regard, further work is necessary to establish whether this activity is still a weak activity in vitro after the attack of LAB by LPcin-YK3.

3.3 | pH and heat stability of LPcin-YK3 peptide

The stability of LPcin-YK3 was studied by measuring antimicrobial activity after heat treatment (100°C for 60 min). The antimicrobial potency of the peptide was determined as the ability to inhibit *S. aureus* growth. The LPcin-YK3 did not appear to be affected by heat treatment of aqueous peptide solutions, resulting in a MIC value of 0.62 μg/mL against *S. aureus* strain under the conditions of heat treatment (Table 2). Furthermore, LPcin-YK3 had the same MIC (0.62 μg/mL) within the limits of a pH (3.7–8.7) solutions, respectively. Meanwhile, the activity of the peptide was reduced at pH 9.4, indicating the increase MIC value of twofold to fourfold (Table 3).

3.4 | Degradation of LPcin-YK3 peptide by *Lactobacillus* species

The effect of the *L. plantarum* subsp. *plantarum* ATCC 10241 and *L. casei* ATCC 393 on the integrity of the 15-mer LPcin-YK3 was

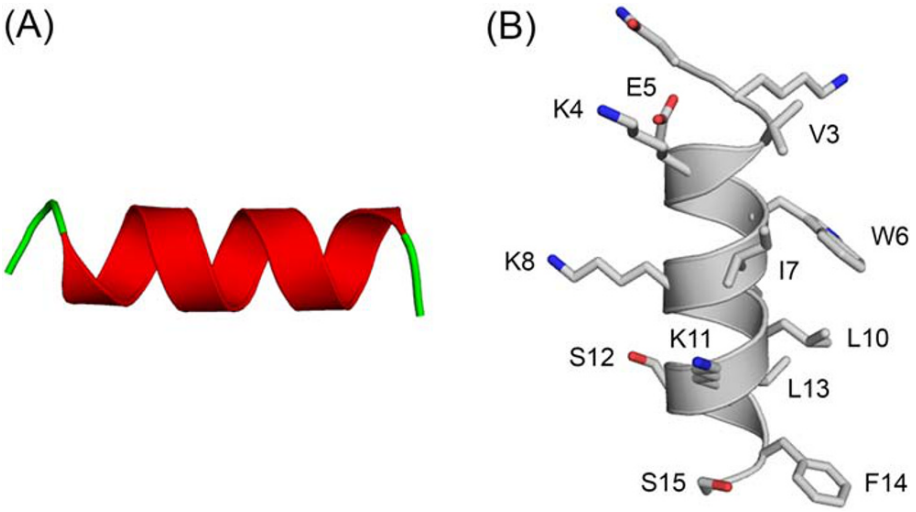

FIGURE 4 In silico prediction of the secondary structure of LPcin-YK3 using PEP-FOLD3 tool (A and B). The secondary-structure element, α-helix of LPcin-YK3 are shown with their corresponding sequences that are indicated as a stick model. The figure was prepared using PyMOL[26]

TABLE 1 Minimum inhibitory concentrations of LPcin-YK3 against bacteria strains

	MIC_{mean} ± SD, μg/mL		
Microorganism	**Ampicillin**	**LPcin-YK3**	**Magainin-2**
Escherichia coli	5.00 ± 0.4	1.25 ± 0.0	>100
Porphyromonas gingivalis	7.50 ± 0.3	0.62 ± 0.0	>100
Propionibacterium acnes	7.50 ± 0.0	1.25 ± 0.4	>100
Staphylococcus aureus	5.00 ± 0.7	0.62 ± 0.5	20.6 ± 1.3
Streptococcus mutans	5.00 ± 0.0	1.25 ± 1.1	62.4 ± 2.8
Lactobacillus acidophilus	0.62 ± 0.0	80.2 ± 1.4	-
Lactobacillus casei	2.50 ± 0.5	>100	-
Lactobacillus coryniformis subsp. *coryniformis*	2.50 ± 0.1	>100	-
Lactobacillus delbrueckii subsp. *lactis*	0.62 ± 0.3	>100	-
Lactobacillus fermentum	0.62 ± 0.0	>100	-
Lactobacillus paracasei subsp. paracasei	2.50 ± 0.2	>100	-
Lactobacillus plantarum subsp. plantarum	0.62 ± 0.6	>100	-
Lactobacillus rhamnosus GG	2.50 ± 0.9	>100	-

Abbreviations: MIC_{mean}, mean of minimum inhibitory concentration values; SD, standard deviation.

TABLE 2 Antimicrobial activity of LPcin-YK3 after heat treatment

Concentration, μg/mL	**5.0**	**2.5**	**1.25**	**0.62**	**0.31**	**0.15**
LPcin-YK3	98.9 ± 1.7	99.2 ± 2.5	98.4 ± 3.1	98.2 ± 0.4	45.6 ± 4.3	6.7 ± 3.2
LPcin-YK3 (heat-treated)	98.2 ± 4.6	98.7 ± 1.1	97.4 ± 0.8	97.5 ± 5.2	3.2 ± 4.4	1.3 ± 8.5

Note. The remaining antimicrobial activity is expressed as the percentage of inhibition of *Staphylococcus aureus* strain, based on optical density (OD) monitoring. Results are expressed as means of three technical replicates ± standard deviation.

evaluated by monitoring the degradation of the peptide by RP-HPLC at pH 7.0 and was compared with that of the *S. aureus* ATCC 25923 which has Gram-positive cell wall with the equivalent of a LAB (Figure 5). Analyses of the samples of LPcin-YK3 treated with either *L. plantarum* (LPT) or *L. casei* (LCS) exhibited very similar results. The chromatographs for both samples showed that the peak corresponding to LPcin-YK3 decreased by approximately 79.3% and 100% after 1 h of treatment, respectively. Subsequent incubation with *L. casei* bacterial strain at longer time points between 1 and 4 h did not show any significant further degradation of this peptide (Figure 5B,D). On the other hand, RP-HPLC analysis of LPcin-YK3 sample treated with *L. plantarum* (LPT) at pH 7.0 revealed an almost complete loss of the peak corresponding to the peptide after just 4 h of incubation, resulting in the two separated products which have different retention times (6.45 and 11.18 min) from that of LPcin-YK3 (Figure 5C).

Whereas, incubation of LPcin-YK3 with non-LAB strain, *S. aureus* (SAR) at pH 7.0 exhibited an entirely different result with those of the LAB strains. Interestingly, when proteolysis occurred within the 1 h, resulting in a loss of just 10.8% of the starting peptide, as evidenced by RP-HPLC analysis (Figure 5A). The RP-HPLC results showed that the peak corresponding to LPcin-YK3 did not decrease any further in size when the incubation proceeded beyond 1 h, indicating that there was no subsequent degradation. The amounts of LPcin-YK3 lost after exposure to three bacterial strains for varying lengths of time were calculated and are shown in Table 4.

Furthermore, we performed an additional study to identify the fragment peaks observed at the *L. plantarum* and *L. casei* strains.

TABLE 3 Antimicrobial activity of bacterial growth inhibition at a variety of pH solutions

	Staphylococcus aureus						
pH values	**3.7**	**4.3**	**5.7**	**6.3**	**7.6**	**8.7**	**9.4**
0 day	98.5 ± 4.6	98.1 ± 2.7	97.3 ± 1.3	98.2 ± 3.8	97.7 ± 2.2	98.9 ± 0.9	94.7 ± 5.2
7 days	98.9 ± 3.5	97.6 ± 0.2	97.4 ± 0.8	98.8 ± 1.9	97.1 ± 3.1	98.6 ± 1.1	89.7 ± 1.2

Note. Results are expressed as means of three technical replicates ± standard deviation. LPcin-YK3 concentration: 0.62 μg/mL.

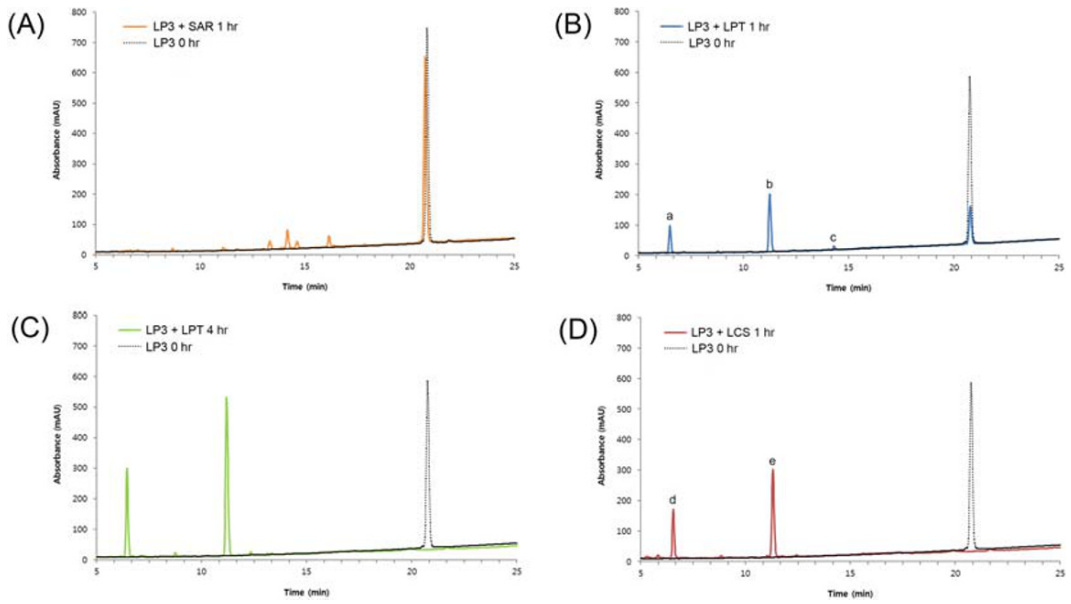

FIGURE 5 RP-HPLC chromatography of LPcin-YK3 (LP3) after incubation with bacterial strains. (A) *S. aureus* (SAR, 1 h); (B) *L. plantarum* (LPT, 1 h); (C) *L. plantarum* (LPT, 4 h); (D) *L. casei* (LCS, 1 h). Lowercase letters a, b, c, d and e indicate the fragment peaks generated by each *Lactobacillus* strain

TABLE 4 Degradation of 15-mer LPcin-YK3 by bacterial strains

Microorganism	Incubation Time, h	Peptide Remaining, %
Control (no bacteria)	0	100
Staphylococcus aureus ATCC 25923	0	100
Staphylococcus aureus ATCC 25923	1	89.2
Lactobacillus plantarum subsp. *plantarum* ATCC 10241	0	100
Lactobacillus plantarum subsp. *plantarum* ATCC 10241	1	20.7
Lactobacillus plantarum subsp. *plantarum* ATCC 10241	4	0
Lactobacillus casei ATCC 393	0	100
Lactobacillus casei ATCC 393	1	0

The eluted peaks (a, b and c of Figure 5B; d and e of Figure 5D) were analyzed via LC-MS/MS equipment and characterized the fragmentation peptides derived from LPcin-YK3 based on the resulting molecular weight. Unfortunately, the two peaks (a and d) were unable to obtain molecular weight data because ionization was not performed despite extensive repeated experiments at the requesting analysis institution. Meanwhile, we analyzed the observed and calculated molecular weights of peaks (b and e) showing the same retention times (11.18 min) obtained from two different *Lactobacillus* strains, finally, the predicted sequences were judged as "SLFS" corresponding to the C-terminal of LPcin-YK3, respectively. In addition, the slightly observed peak c was measured as 596 $[M\text{-}2H]^{2-}$ in the observed molecular weight, and it was judged that the "KEWIKYLKS" sequence matches this result. Details of fragments of LPcin-YK3 are listed in Table 5.

3.5 | Effect of membrane permeability by LPcin-YK3

As further work, it took a visual manner to verify whether LAB will be destroyed or not by the LPcin-YK3 peptide. We examined, therefore, the effect of LPcin-YK3 on the used strains in the degradation assay above using PI uptake assay. The bacteria cells treated by PI in the absence of peptide were used as a negative control. After 20 min of incubation, microscopic observation revealed the presence of PI into the cell membrane treated with the peptide from a concentration of 0.5, 1 and 2 times MIC (*S. aureus*) (Figure 6). *S. aureus* cells were treated with LPcin-YK3 exhibited increased PI fluorescence indicating peptide concentration-dependent membrane permeability, and had very similar to the intensity of the PI fluorescence with that of Triton X-100 used as a positive control, which is widely used to lyse cells to extract protein or organelles, or to permeabilize the membranes of living cells.[27] The PI treated bacteria cells in the absence of peptide, negative control did not see any PI fluorescence. These results suggest that LPcin-YK3 induces cell death of *S. aureus* that undergoes the strong changes in cell morphology. Unlike pathogen *S. aureus* strain, both LAB strains (*L. plantarum* and *L. casei*) revealed that no obvious red fluorescence was found in cells treated with LPcin-YK3, consistent with a degradation of the peak corresponding to

TABLE 5 Fragments of LPcin-YK3 generated by *Lactobacillus* species

HPLC Peak	Predicted Sequence(s)	Molecular Weight, Da		
		Observed	Calculated	Theoretical
b	SLFS	451 $[M-H]^-$	452	452.5
c	KEWIKYLKS	596 $[M-2H]^{2-}$	1,194	1194.4
e	SLFS	451 $[M-H]^-$	452	452.5

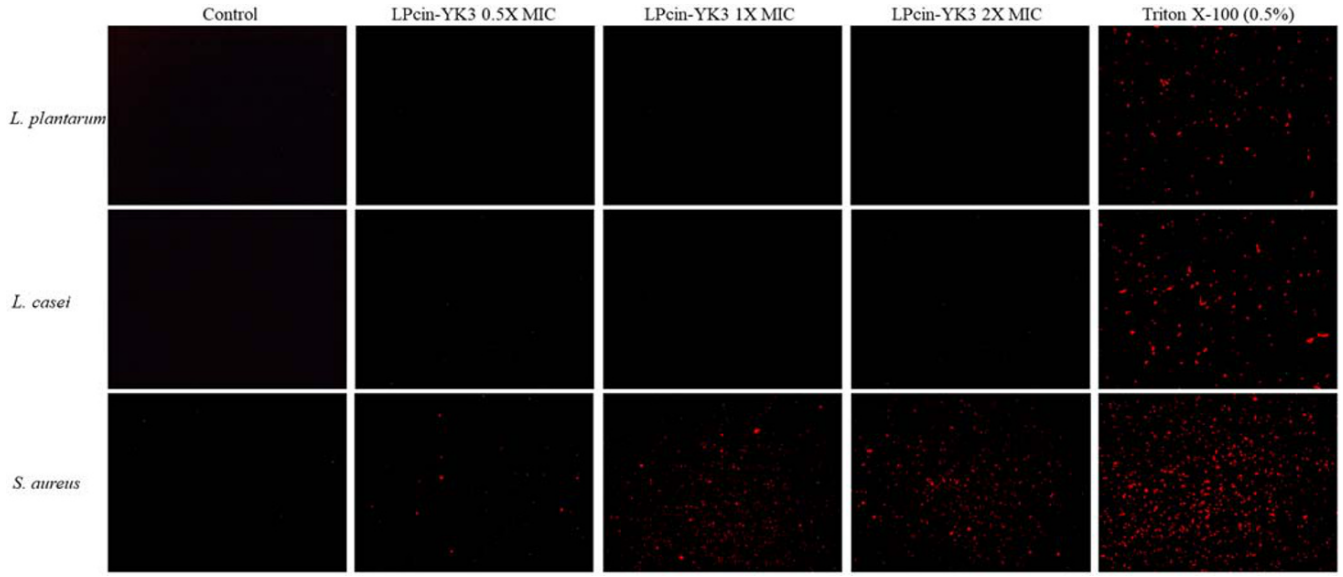

FIGURE 6 Propidium iodide uptake in *L. plantarum*, *L. casei* and *S. aureus* after treatment with LPcin-YK3: Control (left), LPcin-YK3 treatment of 0.31, 0.62 and 1.24 μg/mL (middle), Triton X-100 treatment of 0.5% (right). A fluorescence microscope image of dead cell nuclei stained with propidium iodide is shown, and its image was prepared using the Imaging Software NIS-Elements F 3.00. All tests were repeated three times independently

LPcin-YK3 (Figure 5), although cell walls of these two LAB are known to be similar to a system of *S. aureus*.

4 | DISCUSSION

In the current study, we produced the LPcin-YK3, a natural AMP found in bovine milk component-3 of proteose peptone,[28] using the *E. coli* expression system, and its 3D model revealed as a perfect α-helix consisting of 15 amino acids (Figure 4). Our results showed that the antimicrobial activity of the purified peptide LPcin-YK3 against the tested pathogenic bacteria showed superiority than that of the ampicillin and magainin-2, and the antimicrobial activity of LPcin-YK3 did not show the difference of activity between Gram-positive and Gram-negative strains. Although the antimicrobial activity of LPcin-YK3 to LAB strains suggests that contrary to popular belief, activity to much of LAB is quite a low, resulting in MIC value of over 100 μg/mL (Table 1). This antimicrobial activity of LPcin-YK3 toward LAB strains was consistent with the previous study.[29–32] Interestingly, the bovine lactoferrin that is an iron-binding glycoprotein mainly present in milk and its hydrolysate inhibited the growth of the pathogens in a dose-dependent manner, whereas no significant inhibition was observed on the growth of probiotic strains. It is concluded, consequentially, that the special cell structures or metabolic substances of probiotics may protect the cells from the activities of cationic protein or its hydrolysate.

Based on this study, we focused on the elucidation of the interaction between LPcin-YK3 peptide and specific LAB strains in general. The difference in the antimicrobial activity of LPcin-YK3 between pathogens and LAB strains may be attributed to the effect of the proteolytic systems (proteinases, peptidases, transport systems, etc) of LAB but not pathogenic bacteria. As a piece of evidence to support these statements, the in vitro degradation extent of LPcin-YK3 was determined in each other strains. The results showed that the survival rate of *L. plantarum*-exposed LPcin-YK3 was reduced 4.3-fold than treatment with pathogenic bacteria, *S. aureus*, consistent with the invisibility of the fluorescence corresponding to LAB cells (Figures 5 and 6 and Table 4). In addition, compared with the in vitro environment, the in vivo environment is more complex and contains a large variety of components of the proteolytic pathway. The differences in the biological activity of LPcin-YK3 may be the result of different interactions with these factors.

As described in the Introduction, LAB have been used for centuries as a starter or adjunct culture in dairy fermentations. The

proteolysis by LAB plays an important role in generating peptides and amino acids for bacterial growth and in the formation of metabolites that contribute to flavor formation of fermented products.[7] A detailed understanding of these processes may lead to engineered LAB with improved proteolytic properties. From the structural perspective, proteolysis in dairy LAB has been studied in great detail by the structural methods. Unlike enteric bacteria, LAB have the capacity to transport the long peptides into the cell, a property that is best documented for *L. lactis*.[33] The oligopeptide permease (Opp) plays a primary role in oligopeptide uptake in *L. lactis*. Opp is an ABC transporter, composed of five subunits: nucleotide-binding proteins (OppD and OppF), transmembrane proteins (OppB and OppC) and a substrate-binding protein (OppA), which is anchored to the cell membrane via lipid modification of the N-terminal cysteine.[25] OppA from *L. lactis* binds peptides of widely varying lengths, from four to at least 35 amino residues long, with a lack of specificity for the ligand's amino-acid sequence. Meanwhile, although these peptide transport systems are still poorly characterized in lactobacilli, preliminary experiments suggest that activities similar to those in *L. lactis* are indeed present. Eventually, a comparison of proteolytic systems of different lactococci and lactobacilli has revealed that their components and mode of action may remarkably similar.[34]

In order to structural study on the action mechanism of LPcin-YK3 in the proteolytic system based on the antimicrobial assay, degradation assay and PI staining results in vitro against LAB strains, we carried out the molecular docking using the PatchDock method for interaction between OppA from *L. lactis* and LPcin-YK3 peptide corresponding to receptor and ligand, respectively. The transport receptor molecule, OppA from *L. lactis* was selected based on its potential function that is closely related to the proteolytic pathway in lactobacilli and an analogy of the proteolytic system. Crystal structures are known of the oligopeptide-binding proteins DppA from *E. coli* (specific for dipeptides),[35] OppA from *Salmonella typhimurium* (tripeptides to pentapeptides)[36] and AppA from the *Bacillus subtilis* (possibly nonapeptides).[37] These structures revealed that the specificity for peptides is determined by hydrogen bonds with ligand backbone, and the pocket for ligand binding can accept any side chain, which is the basis for the well-documented lack of specificity for the ligand's amino acid sequence. The bound endogenous peptides (in total 107 peptides) originating from 14 proteins of *L. lactis* were identified with a confidence level of >99%. They ranged in length from seven to 26 amino acids, consistent with the known binding properties of OppA, which is able to bind peptides of a wide variety of lengths, including very long peptides. Furthermore, the most distribution of lengths of endogenous peptides exhibited a length from nine to 11 amino acids and contained at least one isoleucine or phenylalanine.[25] For this reason, we designed the LPcin-YK3 of a length nine amino acids including isoleucine and tyrosine as a ligand molecule of OppA for the molecular docking.

Figure 7 shows a model result on the molecular docking of LPcin-YK3 with the transport receptor OppA. Analogous to other OppAs, the LPcin-YK3 consisting of nine amino acids was observed in the known ligand-binding pocket of OppA from *L. lactis*, presenting a small helix structure (Figure 7A). An OppA-modeled LPcin-YK3 molecule is shown in Figure 7B along with all residues within 4.0 Å that interact with it via hydrogen bonds or van der Waals interactions. The N-terminal Lys-4 of LPcin-YK3 forms a hydrogen bond of less than 3.5 Å with the hydroxyl group of Asp-483 and the side chain of the only acidic Glu-5 of LPcin-YK3 is also engaged in a hydrogen-bonding network with the hydroxyl group of Ser-51 and the nitrogen atom of Gln-69. The C-terminal residues, Lys-11 and Ser-12 of LPcin-YK3 form possible hydrogen bonds with nitrogen atoms of Gln-297 and Asn-276, respectively. The hydrophobic residue Trp-6 is connected by a hydrogen bond to backbone oxygen of Phe-73 and has pronounced

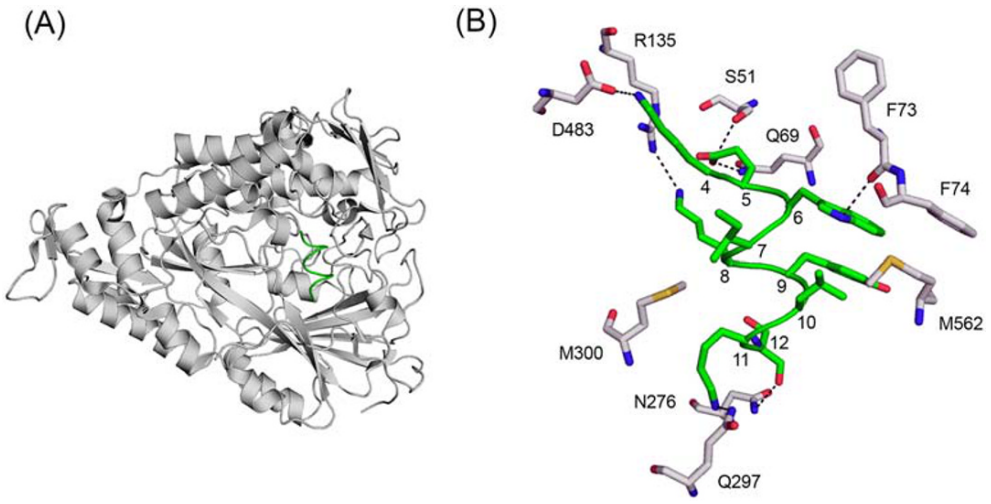

FIGURE 7 Molecular docking of LPcin-YK3 with the transport receptor OppA. (A) The overall structure of monomeric OppA is shown with two α/β-domains in the identical color (gray). The modeled LPcin-YK3 peptide (green) is indicated as a helix model in a potential position for the active site. (B) A view of the ligand-binding site is shown with the modeled LPcin-YK3 molecule depicted as a colored stick model. Labeled residues correspond to OppA and LPcin-YK3 (KEWIKYLKS) structure is numbered the same as Figure 4B. The broken lines indicate putative hydrogen bonds which are observed of less than 3.5 Å from LPcin-YK3 modeled in the active site

hydrophobic interactions together Phe-74 and Met-562 residues. In addition, the Ile-7 and Leu-10 residues of LPcin-YK3 form hydrophobic interactions with a Met-300 and Met-562 residues of OppA, respectively. Slotboom et al[38] had previously reported that the binding mode is determined by a central, well-defined hydrophobic pocket in the binding site of OppA that prefers to accommodate a hydrophobic side chain of the peptide along with hydrogen bonds. In the present study, the major hydrophobic pocket for binding between OppA and LPcin-YK3 may be attributed to the effect of the Trp-6, Ile-7 and Leu-10 residues of LPcin-YK3 based on the model result by docking. Besides, most of the hydrogen bonds exhibited relatively at the N- and C-terminal regions of LPcin-YK3 ligand. Interestingly, although this article was in revision, a degradation study of fragment peaks derived from LPcin-YK3 peptide was performed. The structurally modeled LPcin-YK3 (Figure 7B) sequence as a ligand molecule of OppA is essentially identical with the fragment peak c (Table 5) of LPcin-YK3. These results are consistent with our hypothesis that LPcin-YK3 peptide in LAB proteolytic system could be one of the endogenous substrates of receptor OppA by the putative *Lactobacillus* cell-bound peptidase enzyme. Moreover, these results reveal that the mode of binding may be influenced by both extrinsic and intrinsic factors of proteolytic systems, including the proteolytic pathway, the LAB species, the components exploited, and the fermented environment in which the LAB were grown. This manner of oligopeptide selection may hold in general for Gram-positive organisms that utilize milk proteins as their main source of nitrogen-based on amino acid composition.

In conclusion, the results of this study clearly indicate that bovine-derived peptide, LPcin-YK3 has growth-inhibitory effects in vitro on harmful pathogenic bacteria, maintaining excellent stability. It is noteworthy that this strong activity of LPcin-YK3 which was observed in the pathogenic strains associated with a wide variety of skin diseases[39] was unambiguously identified without separating the Gram-positive and Gram-negative strains. As another part of an antimicrobial potential, our limited data regarding the degradation related to the LPcin-YK3 peptide in LAB proteolytic system, and some earlier finding suggest that the inhibitory inactivity of LPcin-YK3 against LAB strains may be one of the primary pharmacological properties of recombinant peptide discovered in bovine milk, although the possibility of as an endogenous substrate of receptor OppAs and the effect on the growth of lactobacilli strains still remain elusive. It has recently been reported that *Lactobacillus* species from healthy women help to prevent pathogen infection by producing lactic acid and antimicrobial compounds.[40] Further study will be required to establish whether the observed selective activity of LPcin-YK3 actually affects a comparable alteration of the in vivo environment upon the application in a clinical trial along with crystallographic study.

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CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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