ABSTRACT

The LPxTG-motif protein is an important transmembrane protein with high hydrophilicity and stability, as evidenced by its stress tolerance and adhesion ability. In this study, a novel LPxTG-motif protein with esterase activity (LEP) was expressed, and the multifunctional properties such as adhesion properties and esterase activity were also investigated. When cocultured with *Limosilactobacillus reuteri* (L. reuteri) SH-23, the adhesion ability of *L. reuteri* SH-23 to HT-29 cells was improved, and this adhesion was further found relating to the potential target protein Pyruvate kinase M1/2 (PKM) of HT-29 cells. In addition, as a multifunctional protein, LEP can promote the hydrolysis of bovine milk lipids with its esterase activity, and the activity was enhanced in the presence of Zn$^{2+}$ and Mn$^{2+}$ at pH 7. Furthermore, the polyunsaturated fatty acids (PUFA) such as linoleic acid and eicosapentaenoic acid were found to increase during the hydrolyzing process. These unique properties of LEP provide a comprehensive understanding of the adhesion function and PUFAs releasing properties of the multifunctional protein derived from *L. reuteri* SH-23 and shed light on the beneficial effect of this *Lactobacillus* strain on the colonization of the gastrointestinal tract.

**Key words:** *Lactobacillus*, LPxTG-motif protein, adhesion, esterase properties

INTRODUCTION

Gut microbiota homeostasis has a significant effect on human health, and probiotics are a key factor in maintaining the optimal composition of the gut microbiota. In recent years, studies have found a strong link between probiotic colonization and gut microbiota homeostasis in the gastrointestinal tract (GIT; Saxami et al., 2012; Wang et al., 2021). Surface components of lactic acid bacteria (LAB) such as surface proteins, extracellular polysaccharides, and other adhesion-related molecules play an important role in intestinal adhesion and have been extensively studied, while the multifunctional properties of surface proteins were rarely explored.

The surface proteins (S-layer proteins) are the proteins representing the outermost layer of the cell wall and account for 10–15% of the total proteins in bacterial cells (Yadav et al., 2017). In addition to some common functions of cell surface components, such as cytoprotection, cell-specific topologies formation, cell wall metabolism, catabolism and nutrients absorption, communication, and substrates or hosts binding, they also play an important role in the regulation of adhesion and immune responses in intestinal tissues (Zhou et al., 2010; Alp et al., 2020). It has been found that when S-layer proteins are removed from the LAB, their adhesion to the target cells was also reduced (Kos et al., 2003; Garrote et al., 2004; Frece et al., 2005; Jakava-Viljanen and Palva, 2007; Hynönen and Palva, 2013). S-layer proteins have various mechanisms to promote LAB colonization in the intestine, such as the binding to epithelial cells and extracellular matrix components (Hynönen et al., 2002; Ávall-Jääskeläinen et al., 2003). LPxTG-motif protein is a special S-layer protein that located on the surface of LAB that can be recognized by Sortase A and anchored to the peptidoglycan of the cell wall to enhance bacterial adhesion (Zhang et al., 2015; Ton-That et al., 2000; Mathiesen et al., 2020; Lu et al., 2023). The co-adhesive effect of the LPxTG-motif protein improves the colonization properties of LAB in the GIT, prolonging the residence time of probiotics in the GIT and enhancing their probiotic effect on the host (Chow, 2002). In recent years, the multifunctional properties of LAB S-layer proteins have been gradually discovered. Kim et al. found that the S-layer proteins of LAB in Kefir yogurt have both anti-inflammatory properties and alleviate obesity caused by a high-fat diet (Kim et al., 2021). Alp et al. found that S-layer
proteins have endopeptidase activity and promote aggregation and adhesion of LAB to the host (Alp et al., 2020). Some research also found the S-layer proteins of Lactobacillus acidophilus ATCC 4356 have hydrolase activity (Prado Acosta et al., 2008).

Lactic acid bacteria with high esterase activity is of great importance for yogurt fermentation and human health. It can replace the strains of weak enzyme activity in the human intestinal microflora (García-Cano et al., 2019, 2020) and alleviate the esterase or lipase deficiency in the digestive tract (Pezzilli, 2009). Lactic acid bacteria are not only one of the most widely used fermenters in industry but also a good source of esterase (Esteban-Torres et al., 2016). Most of the LAB are weak esterolytic strains and their lipase systems are not as attractive as their protein hydrolysis systems. Lactic acid bacteria with high esterase activity can catalyze the hydrolysis of milk fat during cheese ripening, producing various flavor substances and improving the quality of fermented dairy products (Holland et al., 2005; Esteban-Torres et al., 2014). Furthermore, PUFA produced in the esterolysis process can improve human nutritional absorption, metabolic disorders, cholesterol, and malnutrition (Kris-Etherton and Fleming, 2015).

In this study, the adhesion properties and esterase activity of the transmembrane LPxTG-motif protein derived from Limosilactobacillus reuteri SH-23 will be detected, and the unique adhesion and esterase activity of the LEP were also investigated. Meanwhile, the hydrolysates of the fatty acids released during the LEP treatment will also be obtained for the application potential in the fermented food related gastrointestinal health and diseases treatment.

MATERIALS AND METHODS

Strains

Lactobacillus reuteri SH-23 (DSMZ 8533) was stored in China General Microbiological Culture Collection Center. Escherichia coli (E. coli) BL21 (DE3) competent cells were obtained from the Vazyme (Nanjing, China). HT-29 cells were purchased from FuHeng Biotechnology (Shanghai, China). All strains were preserved in 25% (vol/vol) glycerol at −80°C before use.

Growth Conditions

Lactobacillus reuteri SH-23 was cultured in MRS broth at 37°C for 12 h, and LEP expression was carried out using E. coli BL21 (DE3) competent cells and the pET-28a vector in our laboratory. HT-29 cells were cultured in a humidified 5% CO₂ atmosphere at 37°C.

Medium used for HT-29 cells cultured is DMEM/F12-Dulbecco’s Modified Eagle Medium (Gibco, CA, USA).

LEP Recombinant

E. Coli Strain Construction. The LPxTG gene (A5VKB5) of L. reuteri SH-23 consists of a 2,175 bp open reading frame that encodes a polypeptide of 725 amino acids (NCBI: GenBank: EDX43010.1). The total DNA from L. reuteri SH-23 was isolated using the EasyPure Quick Gel Extraction Kit method (Transgen, Pecking, China). Primers were designed using CE Design (italicized words are digestion sites) and the following PCR-amplified primers were used:

Forward: 5′-gttgtgtgtgtgtgtgtgtgcteqaqGATATGACTGGGACAACAGTTAATGG;

Reverse: 5′-cagcaaatgggtcgcggatccTTAAGCATGTTTACGCTTGCG

The PCR amplification conditions were: pre-denaturation at 95°C for 3 min, denaturation at 95°C for 15 s, annealing at 56°C for 15 s, extension at 72°C for 2 min, extension at 72°C for 5 min, repeated for 35 cycles. The amplified target genes were purified using an agarose gel DNA purification kit (Transgen, Pecking, China). Thereafter, the fragments were digested and cloned into pET-28a digested by XhoI and BamHI (Takara Biomedical Technology, Beijing, China), expressed in E. coli BL21 (DE3) and grown in Luria-Bertani (LB) medium. The recombinant strain was sequenced for base sequence (Sangon Biotech, Shanghai, China) and named as the LEP strain.

LEP Expression and Purification

The LEP strain was grown in LB medium containing kanamycin (50 μg/mL). When the OD value of the strain medium was 0.6 ± 0.01 at 600 nm, the LEP expression condition in the recombinant strain was optimized: induction temperature (25°C, 30°C), the concentration of the inducer IPTG (0, 0.1, 0.4, 0.7, 1 mM), and induction time was 4 h. After that, the crude proteins in the membrane layer were isolated by the ultrasonic treatment (30 min, 300 W) and proteins in the supernatant were obtained after centrifugation (4°C, 10,000 rpm, 30 min) and analyzed by the SDS-PAGE (10% polyacrylamide). The LEP was purified in imidazole buffer (0, 10, 50, 75, 100, 200 mM) elution using HisTrap columns of ProteinIso Ni-IDA resin (TransGen, Beijing, China). The purified LEP was verified by SDS-PAGE (10% polyacrylamide) and the
LEP Bioinformatics Analysis

Homology comparison and analysis of LEP were performed using Geneious, SWISS, uniport and Pfam websites. ProtParam (http://web.expasy.org/protparam/) was used to predict the primary structure (physicochemical property analysis, hydrophilic/hydrophobic analysis, aliphatic index, instability index, and so on) of LEP. The hydropathy plot was predicted using ProtScale (http://web.expasy.org/protscale/). Based on statistical analysis of the TMBase database, the TMpred method predicts the protein transmembrane region. The Multiple Em for Motif Elimination (MEME) server (http://meme-suite.org/) was used to predict the conserved motif of LEP. The secondary structure of LEP was predicted by the SOPMA server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_sopma.html). Pfam (http://pfam.xfam.org/) prediction domain. The 3D structure of LEP was obtained from the UniProt (https://www.uniprot.org/).

Confirmation of LEP Binding to HT-29 Cells Surface Proteins

The principle of protein interactions and His-Tag Isolation and Pulldown technique was used to identify the cell surface proteins interacting with LEP and HT-29 cells. Dynabeads magnetic beads were thoroughly suspended in the vial and 50 μL (2 mg) Dynabeads magnetic beads were transferred to a microcentrifuge tube, which was placed on the magnet for 2 min, then aspirated and the supernatant was discarded. The LEP samples containing histidine-tagged LEP were prepared in a total volume of 700 μL of 1 × BWB (2 × BWB:100 mM Sodium Phosphate, pH 8.0, 600 mM NaCl, 0.02% Tween-20), and added to Dynabeads magnetic beads before resuspending. The samples were incubated on a roller for 5 min at room temperature, then the tubes were placed on the magnet for 2 min and the supernatant was discarded. The beads were washed 4 times with 300 μL 1 × BWB by placing the tube on a magnet for 2 min and discard the supernatant.

Meanwhile, the lysed HT-29 cells lysate were prepared in a total volume of no more than 700 μL of 1 × PDW (2 × PDW: 6.5 mM Sodium phosphate, pH 7.4, 140 mM NaCl, 0.02% Tween-20). The sample (prepared in 1 × PDB) was added to the above-prepared magnetic beads and resuspended. The tubes were incubated on a roller for 10 min at room temperature and then placed on a magnet for 2 min and the supernatant was discarded. The tubes were placed on a magnet for 2 min and the beads were washed 4 times with 300 μL of 1 × BWB resuspension and the supernatant was discarded. Resuspended the beads thoroughly between each washing step. The suspension was incubated on a roller for 5 min after adding 100 μL of His-elution buffer (300 mM Imidazole, 50 mM Sodium phosphate, pH 8.0, 300 mM NaCl, 0.01% Tween-20), and then the beads were collected from the tube wall using a magnet. The supernatant containing the eluted histidine-tagged protein and its interacting proteins is transferred to a clean tube. Finally, SDS-PAGE (10% polyacrylamide) was used for the binding protein isolation and visualized by Coomassie brilliant blue staining.

LC-MS/MS Analysis of LEP Complexed with HT-29 Cells Protein

After SDS-PAGE detection, the gum strips were enzymatically digested (Wisniewski et al., 2009), desalted (Zheng et al., 2016) and dried. LC-MS/MS analysis was carried out on an UltiMate 3,000 nano ultra-performance liquid chromatography tandem with a Q-Exactive plus high-resolution mass spectrometer (Qing et al., 2017; Zhao et al., 2018). The separation was performed using a nano-HPLC liquid phase system, UltiMate 3000 RSLCnano (Thermo Fisher Scientific, Massachusetts, USA). Nano-HPLC buffer A was 0.1% formic acid in water and nano-HPLC buffer B was 0.1% formic acid in acetonitrile. The chromatography column trap column (100 μm × 20 mm, RP-C18, Agilent) was equilibrated with 100% buffer A (3 μL/min). The samples were loaded by the autosampler, combined with a trap column, and then separated by an analytical column (75 μm × 150 mm, RP-C18, New Objective, USA) at a flow rate of 300 μL/min. Samples were washed through the gradient mobile phase with blank solvent for 30 min. The enzymatic digestion products were separated using capillary HPLC and analyzed by Q-Exactive plus mass spectrometry (Thermo Fisher Scientific Massachusetts, USA). For the qualitative protein analysis in experimental samples, ProteomeDiscover 2.5 and UniProt-Human/UniProt-Lactobacillus reuteri (strain DSM 20016) were used.

The Effect of LEP on the Expression Level of HT-29 Cells Surface Protein

LEP sample was added to 6-well plates containing HT-29 cells and incubated for 4 h. The total RNA
of HT-29 cells was extracted by the Trizol method, and RNA concentration were determined by Nano-300 Micro-Spectrophotometer (ALLSHENG, Hangzhou, China). After that, the reverse transcription system (4 μL UEiRis II RT MasterMix, 1 μg template RNA, 1 μL dsDNase, RNase-free water to 20 μL) was gently mixed and centrifuged (the liquid settled to the bottom). The following conditions were performed on the T100™Thermal Cycler (BIO-RAD, California, USA): incubation at 37°C for 2 min, 55°C for 10 min, and 85°C for 10 s. The obtained product was stored at −40°C. RT-qPCR was performed using the kit PerfectStart Green qPCR SuperMix (Transgen, Pecking, China) and the primers used for the experiments are described in Supplemental Table S1. The 2-ΔΔCt method was used to assess mRNA expression levels.

**Molecular Docking and Analysis of LEP**

FAST sequences of LEP (A5VKB5) were downloaded from the UniProt database and homology modeling was performed using α-FOLD. The protein structures of CSNK1D (PDB ID: 3UYS), HSPA8 (PDB ID: 4H5W), ANXA2 (PDB ID: 6TWY), and PKM (PDB ID: 6NUB) were obtained from the PDB database. Protein molecular docking was performed using Zdock, and interaction pattern analysis was performed using Pymol 2.3.0.

**Cytotoxicity of LEP to HT-29 Cells**

HT-29 cells were inoculated into 96-well plates and pre-cultured in a cell culture incubator at 37°C and 5% CO2 for 24 h. Then, LEP (100 μL) of a final concentration of 0, 20, 40, 60, 80, 100, 120, 140 μg/mL were added to the cells and incubated for 12 h. After that, 10 μL of Cell Counting Kit-8 (Dojindo, Shanghai, China) reagent was added to each well. After 4 h of incubation, the absorbance at 450 nm was measured with Tecan Infinite M200 Pro (Tecan Group, Switzerland) for the cytotoxicity of LEP to HT-29 cells.

**The Adhesion Experiment of LEP to HT-29 Cells**

HT-29 cells were preincubated with LEP (0, 20, 40, 60 μg/mL) in 6-wells and 12-well plates for 12 h (37°C, 5% CO2). Thereafter, 20 μL FITC-stained (10 μM) L. reuteri SH-23 was added to 12-well plates and 6-well plates containing HT-29 cells and incubated for 2 h. The 12-well plates and 6-well plates were washed 3 times with PBS to remove nonadherent bacteria. The adhesion of L. reuteri SH-23 to HT-29 cells in 6-well plates was observed and photographed by an inverted fluorescence microscope. Finally, HT-29 cells and L. reuteri SH-23 in 12-well plates were treated with trypsin and the fluorescence intensity was measured with Tecan Infinite M200 Pro to record the fluorescence value A1 after PBS washing. The initial fluorescence intensity A0 (excitation wavelength 485 nm; emission wavelength 538 nm) of L. reuteri SH-23 was also measured (Tecan Group, Switzerland). The adhesion rate of bacteria was calculated according to the following formula:

\[
\text{Adhesion rates} \% = \frac{A_1 - A_0}{A_0} \times 100\%.
\]

**Effect of pH and Metal Ions on LEP Esterase Activity**

First, 0.63 mL of solution A (3.0 mg of p-nitrophenyl palmitate dissolved in 1.0 mL of isopropanol) was added to 5.67 mL of solution B (1,000 mL of water with one drop of TritonX-100, pH = 4, 7, 10) in a 10 mL tube and thoroughly mixed, made as solution C and kept at 37°C for 5 min. After that, solution C (0.9 mL) and the enzyme solution (200 μg/mL, 0.1 mL) were mixed together into the centrifuge tube and reacted at 37°C for 30 min. Finally, 95% ethanol (0.5 mL) was added to terminate or stop the reaction, and the absorbance was measured at 410 nm by Tecan Infinite M200 Pro (Tecan Group, Switzerland). The average value of each sample was measured 3 times. Meanwhile, metal ions (K+, Ca2+, Cu2+, Mn2+, Zn2+, 1 mM) were added to solution B at pH = 7 to determine the effect of metal ions on enzyme activity.

**Effect of LEP on Hydrolysis of Milk Fat**

In the experimental group, milk (30 mL, Ningbo Dairy Group) was sterilized at 80°C for 15 min, then LEP was added to make the final protein concentration 100 μg/mL and hydrolyzed at 37°C for 1 h. Without changing other conditions, the inactivated protein was added to the milk sample as the control group. The hydrolyzed milk (30 mL) was transferred to a flask (250 mL) and mixed with pyrogallic acid (100 mg), 95% ethanol (2 mL), water (4 mL) and ammonia (5 mL). The flask was placed in a water bath (75°C) for 20 min, and shaked every 5 min. After the hydrolysis, the sample was mixed with 95% (10 mL) ethanol, and the hydrolysate in the flask was transferred to the separating funnel. The flask and the plug were washed with an ether-petroleum ether mixture (1:1 vol/vol, 50 mL), and the rinse solution was added to the separatory funnel. After shaking for 5 min, the extract of the ether-petroleum ether mixture was collected in a flask (250 mL). The hydrolysate was repeatedly extracted 3 times.
followed the former steps. Finally, the separation funnel was washed with the ether-petroleum ether mixture, and the liquid was collected and the residual solvent in the sample bottle was dried by liquid nitrogen and stored at 4°C overnight.

In the saponification experiment, the collected sample was mixed with KOH (6%) dissolved in methanol and water (4:1 vol/vol). Saturated salt water (1 mL), hydrochloric acid (0.5 mL, 12 M), and a chloroform-hexane mixture (2 mL, 1:4 vol/vol). After centrifugation, the upper liquid was transferred to a new tube (10 mL), and mixed with chloroform and hexane (2 mL, 1:4 vol/vol) for 2 min shaking. The above steps were repeat twice. After that, the centrifuge tube was washed once with distilled water (2 mL) and the supernatant was transferred to the sample bottle (4 mL) after shaking for 2 min. The residual solvent in the sample bottle was dried by nitrogen and stored at 4°C overnight.

During the methylated procedure, a methanolic solution of BF₃ (0.5 mL) was added to the samples and methylated in a water bath at 60°C for 1 h, followed by cooling in a centrifuge tube (10 mL). After that, saturated NaCl solution (2 mL) and hexane (2 mL) were added to the sample, and transfer the organic phase to a new centrifuge tube, and repeat once. Next, the sample was centrifuged with chloroform:hexane (1:4 vol/vol) and remove the upper organic phase. After the water removed from the organic phase with Na₂SO₄, the organic phase was blown dry with nitrogen, and 0.5 mL of hexane (chromatographic purity) was used to reconstitute the solution. The obtained fat acids solution was collected for the GC-MS analysis, and the detection conditions were as follows: column TG-5MS (30 m × 0.25 mm × 0.25 μm), heating program was 100°C for 13 min, 10°C/min to 180°C for 6 min, 1°C/min to 200°C for 20 min, 4°C/min to 230°C for 10.5 min, inlet temperature is 290°C, carrier gas flow rate was 1.2 mL/min, shunt ratio was no shunt, mass spectrometry conditions were ion source temperature 280°C, transmission line temperature was 280°C, scanning range 30–400 amu, ion source EI 70 eV.

**Statistical Analysis**

All data were presented as means ± standard deviations (SD) and analyzed using GraphPad Prism 8.0 program (GraphPad Software, San Diego, Canada). One-way ANOVA was used to compare data from more than 2 groups, followed by Tukey’s multiple comparison tests. The adjusted \( P < 0.05 \) was considered statistically significant. The Figures were created and edited using CorelDRAW 2020 and the Biorender website.
**Figure 1.** Cloning, expression, induction, and purification of LEP. (A) Recombinant expression plasmid construction map; (B) The PCR amplification product of the target LPxTG gene (A5VKB5) of *L. reuteri* SH-23; (C) Analysis of LEP induced by IPTG with SDS-PAGE; (D) The SDS-PAGE of recombinant strains after IPTG induction; a is SDS-PAGE of bacteriophage precipitation, b is SDS-PAGE of the supernatant; (E) Gradient elution of LEP with different concentrations of imidazole.
**Protein Interaction Analysis**

After that, the pulldown analysis was performed using the LC-MS/MS approach to identify the interaction proteins on the surface of the HT-29 cells. The results showed that a total of 240 proteins were identified as essentially belonging to membrane proteins, protein N/C-terminal binding proteins, and cadherins. Four HT-29 cells surface proteins CSNK1D, HSPA8, ANXA2, PKM are proved association with surface adhesion and protein binding functions, and these proteins were also found in greater abundance (Supplemental Table S5, Figure 3B). These 4 proteins accounted for 0.838% of the total interacting proteins, with PKM accounting for the most at 0.4679%, followed by SHPA8 at 0.2779%, CSNK1D and ANXA2 at 0.031% and 0.0595%, respectively (Figure 3C). Furthermore, RT-qPCR was also confirmed the transcription levels of the screened 4 proteins (Figure 3A). Results showed that ANXA2 had the highest relative expression level (6.45), and HSPA8 expression was similar with ANXA2 (5.32). The ex-

**Figure 2.** Bioinformatics analysis of the target LEP by the AA sequence of the protein. (A) Hydropathy plot of LEP by the Kyte-Doolittle model; (B) Tertiary structure analysis of LEP by the Homology/analogY Recognition Engine V2.0 (PHYRE2) server; (C) The transmembrane domain of LEP by TMpred.
Figure 3. Analysis of proteins interacting with LEP in HT-29 cell surface proteins. (A) Relative expression of ANXA2, CSNK1D, PKM, and HSPA8 in HT-29 cells surface proteins after interaction with LEP (PGK1 is the internal reference gene), different letters indicate a significant difference ($P < 0.05$); (B) The SDS-PAGE analysis of LEP-HT-29 surface cells by the pulldown assays. (C) Screened target-interact proteins by the LC-MS/MS analysis. (D) LEP was molecularly docked to ANXA2 (a), CSNK1D (b), HSPA8 (c), and PKM (d). Letters represent different proteins docked with LEP (the green part represents LEP), where the numbers represent structures of the protein (1), Hydrophobic interactions formed by protein-protein interactions (2), Hydrogen bonds formed by protein-protein interactions (3), respectively.
Figure 4. Adhesion properties of the LEP in vitro. (A) Cytotoxicity assay of the LEP proteins on HT-29 cells; (B) Adhesion rate of L. reuteri SH-23 to HT-29 cells. (C) Actual fluorescence graph. Letters represent different concentrations of LEP (a: 0 μg/mL; b: 20 μg/mL; c: 40 μg/mL; d: 600 μg/mL), and the numbers represent the scene (1: fluorescent image; 2: bright field image; 3: confocal image). ***P < 0.001, **P < 0.01, P < 0.05.
Figure 5. The LEP enzyme activity determination with different methods. (A) Effect of pH on LEP enzyme activity; (B) Effect of metal ions on LEP enzyme activity (LEP concentration: 200 μg/mL, T: 37°C, t: 30 min); (C, D) Milk fatty acids (c, C_{16}; d, C_{18-22}) released during the LEP treatment.
pression level of CSNK1D (4.25) and PKM (4.33) was slightly lower without a significant difference.

ZDOCK was used to detected the binding property of LEP with the above 4 surface proteins, and the 4 docking models with the lowest energy were selected, which are LEP-ANXA2 at 1.341.536, LEP-CSNK1D at 1.403.516, LEP-HSPA8 at 1.398.136, and LEP-PKM at 1.436.145, respectively. The docking results showed (Figure 3D) that some AA residues in the protein interaction surface can form hydrophobic interactions, van der Waals forces and hydrogen bonds. Among the large number of hydrogen bonds formed LEP-HIS-27-ANXA2-ASP-524, LEP-PHE-21-ANXA2-PHE-470, LEP-ASN-288-CSNK1D-GLU-238, LEP-PHE-21-HSPA8-TYR-183, LEP-LEU-19-HSPA8-ASP-186, LEP-LEU-17-HSPA8-GLN-376, LEP-ASP-261-PKM-THR-409, LEP-GLN-589-PKM-TRP-515 hydrogen bond lengths are: 2.3 Å, 2.5 Å, 2.3 Å, 1.9 Å, 2.2 Å, 2.5 Å, 2.4 Å, 1.5 Å, respectively, which are much smaller than conventional hydrogen bonds.

**Adhesion Properties of the LEP In Vitro.**

HT-29 cells activity was measured by Cell Counting Kit-8 (Figure 4A). The results showed that the cell survival rate of all groups was more than 96%, demonstrating that LEP had no toxic effect on HT-29 cells in low doses (<60 μg/mL) and high doses (>60 μg/mL). In the adhesion property analysis, the adhesion rate of *L. reuteri* SH-23 to HT-29 cells was increased after LEP treatment compared with the control group (Figure 4B). As the LEP concentration increased, the brightness of the fluorogram gradually increased and the amount of adhesion increased significantly. When the LEP concentration was 60 μg/mL, the adhesion rate was significantly higher than in other groups (*P* < 0.001, 6.05%), as confirmed by the fluorescence staining microscopic observation method (Figure 4C).

**LEP Enzyme Activity Determination**

To determine the multifunction of LEP, the effect factors related to the enzyme activities of LEP, such as pH and ions, were determined. When the pH was 7, the enzyme activity was 71.83 U/mg, which was approximately 100% of the enzyme activity (Figure 5A). Zn^{2+}, Mn^{2+}, Ca^{2+}, K^{+} increased enzyme activity, and Zn^{2+} and Mn^{2+} are the best coenzyme ion for LEP (Figure 5B). In addition, the effect of LEP on milk lipid hydrolysis was studied in vitro (Figure 5C, Figure 5D). The fatty acids released during the LEP treatment were significantly higher than in the control group, including methyl caproate and stearic acid. It also promoted the formation of PUFA, such as tetradecenoic acid, linoleic acid, trans linoleic acid, arachidonic acid, wood tar acid, and eicosapentaenoic acid.

**DISCUSSION**

LEP is a surface protein of gram-positive bacteria that has a cell wall sorting signal consisting of LPxTG structure, a hydrophobic domain, and a positive tail (Siegel et al., 2016). This unique structure has improved the adhesion properties of the bacteria in the GIT environment (Xu et al., 2021). In our study, we identified a novel esterase activity protein with the adhesion-related LPxTG-motif derived from *L. reuteri* SH-23, and the biological information of LEP was proven to be a surface protein with a high stability coefficient and transmembrane structural domain, which was consistent with the properties of the LAB strains surface proteins and was further confirmed by in vitro adhesion analysis. The uniformly alternating distribution of hydrophilic and hydrophobic AA residues of LEP is similar to the integral membrane protein-2 associated with gastrointestinal adhesion in the surface protein of *Lactobacillus plantarum* CGMCC1258 (Liu et al., 2011). Previous studies have found that if MUBs lacking the LPxTG region at the C-terminal, *L. reuteri* could not be anchored to the cell wall, and the cells had a low binding rate and self-aggregation ability (MacKenzie et al., 2010), revealing the importance of the LPxTG region in the C-terminal structure of surface proteins. In this study, a similar result was found that LEP enhances the adhesion of *L. reuteri* in HT-29 cells.

Pulldown and RT-qPCR results also showed that adhesion-related proteins (HSPA8, ANXA2, CSNK1D, PKM) will be overexpressed in HT-29 cells after LEP treatment. The protein docking results show that LEP can interact with the 4 surface proteins and the residues around the protein-protein interaction interface can form a large number of hydrophobic interactions and hydrogen bonds. These noncovalent bonds can help stabilize the protein-protein complexes for better binding. It suggested that LEP might promote the expression of adhesion-related protein genes in the HT-29 cells surface proteins, which indicated that multifunctional proteins containing LPxTG structures on the surface of LAB were helpful for adhesion when binding to ANXA2 or HSPA8 protein on the HT-29 cells surface. In the meantime, protein ANXA2 is a member of the annexin family that plays a role in the regulation of cells growth and signal transduction pathways. Some research also found the ANXA2 deletion inhibited Rickettsia attachment to the endothelial cells in vitro and blocked Rickettsia adherence to the luminal surface of blood vessels in vivo (He et al., 2019). When ANXA2 was blocked with recombinant pneumococcal surface adhesin A, pneumococci had a negative effect on the adhesion of ANXA2-transfected human embryonic kidney cells (Hu et al., 2021). Molecular chaperone Hsp70
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(HSPA8) is a member of the heat shock protein family that can protect nascent polypeptides from misfolding and aggregation due to its unique ability to interact with non-native proteins. Hsp70 is a molecular chaperone that binds small molecules such as proteins and peptides mainly through secondary interactions with the peptide backbone, hydrogen bonds and side chains (Gaur and Klotz, 2004). Although classified as intracellular proteins, the family of heat shock proteins (Hsps) has been identified on the surface of several mucosal pathogens with cell adhesion and immune regulation properties. In this study, PKM was the potential target protein that could bind to the LEP in the HT-29 cells adhesion model.

The novel LEP is annotated to have potential esterase activity but has rarely been studied, and this property has never been demonstrated in previous studies. The conditions identified in this study for exerting optimal esterase activity of LEP are the same as most enzymes in LAB. Furthermore, LEP has been shown to have potential esterase activity in the milk fat hydrolyzation, which can increase fatty acid content, and promote the production of PUFA. It has been found that PUFA can affect the adhesion of LAB to the intestinal surface and low doses of linoleic acid and arachidonic acid have been shown to help *Lactobacillus casei* Shirota grow and adhere to Caco-2 cells (Kankaanpää et al., 2001, 2004). Minor changes in hydrophobicity do not explain the observed effects of free PUFA on bacterial adhesion to mucus and epithelial cells. However, it does suggest that the fatty acid composition of probiotics may influence the process of adhesion to microorganisms by affecting other factors such as the fluidity of bacterial membranes and membrane-lipopeptide interactions. The adhesive properties of LEP contribute to the colonization of LAB in the intestinal tract, which can exert the esterase activity of LAB in the intestinal environment. Meanwhile, due to its esterase properties, LAB can hydrolyze lipids and free PUFA, which can be absorbed by LAB in GIT, thus promoting the colonization of LAB in the GIT.

Esterase in LAB not only catalyze the hydrolysis of milk fat glycerides to release small molecular fatty acids, but also synthesize esters from glycerides and alcohols through transferase reactions to produce important flavor compounds in fermented dairy products (Holland et al., 2005). Furthermore, as a carboxylic acid hydro-lase, esterase can catalyze the hydrolysis and synthesis of water-insoluble long-chain acyl glycerol (Angonesi Brod et al., 2010). It has been found that LEP can catalyze the hydrolysis of milk fat, but whether it can synthesize esters with flavor has yet to be proven.

In summary, LEP from *L. reuteri* SH-23 is a multi-functional surface protein with adhesive properties as well as novel esterase activity. It promotes LAB adhesion to HT-29 cells by binding to the ANXA2 protein on the cell surface of the target cells. Meanwhile, the esterase property of LAB enables it to hydrolyze lipids and release free PUFA in the intestinal tract environment. The unique features of LEP can provide us a better understanding of the novel LAB’s surface proteins, which provides new evidences that LPxTG-motif proteins have novel multifunctional properties.

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**ORCIDS**

Qianwen Ye © https://orcid.org/0000-0009-3917-4124
Lifeng Lao © https://orcid.org/0000-0002-3637-5598
Daodong Pan © https://orcid.org/0000-0002-5299-644X
Hua Yang © https://orcid.org/0000-0002-5709-3952
Zhen Wu © https://orcid.org/0000-0002-3607-6440