

## Original Research Article

# Expression and antimicrobial activity of the recombinant bovine lactoferricin in *Pichia pastoris*

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## ABSTRACT

Lactoferricin, a multifunctional peptide located in the N-terminal region of lactoferrin, has a broad-spectrum bacteriostatic activity. It is a promising candidate as a food additive and immune fortification agent and does not have the risks associated with drug residues and drug resistance. First, we performed promoter and host cell screening to achieve the recombinant expression of lactoferricin in *Pichia pastoris*, showing an initial titer of 19.5 mg/L in *P. pastoris* X-33 using P<sub>AOX1</sub> promoter. Second, we constructed a 0030- $\alpha$  hybrid signal peptide by fusing the 0030 signal peptide with the pro-sequence of  $\alpha$ -factor secretory signal peptide. This further increased the production of lactoferricin, with a titer of 28.8 mg/L in the fermentation supernatant in the shaking flask. Next, we increased the expression of lactoferricin by fusing it with anionic antioxidant peptides. The neutralization of positive charges yielded a titer of 55.3 mg/L in the shaking flask, and a highest titer of 193.9 mg/L in a 3-L bioreactor. The antimicrobial activity analysis showed that recombinant-expressed lactoferricin exhibited potent antibacterial activity against *Escherichia coli*, *Bacillus subtilis*, and *Staphylococcus aureus*. This study provides a reference for the construction of microbial cell factories capable of efficiently synthesizing antimicrobial peptides.

## 1. Introduction

Antimicrobial peptides (AMPs) are small peptides that exhibit broad-spectrum antimicrobial activities against bacteria and fungi [1,2]. These unique naturally occurring antimicrobial compounds can be used as antioxidants, food additives, and immune fortification agents regardless of the problems related to drug residues and drug resistance [3]. AMPs have been identified in diverse organisms, from bacteria to plants to humans. Some of the most well-known AMPs are lactoferrin peptides, which are short-functional peptides released from lactoferrin by the action of proteolytic enzymes in the digestive environment [4]. It has been reported that lactoferrin peptides exhibit antibacterial effects that are 200–400-fold more potent than lactoferrin [5].

Many antimicrobial lactoferrin peptides have already been isolated and characterized, and the N-terminal peptides of lactoferrin have

stronger antibacterial activities than its C-terminal peptides [6]. Lactoferricin, a multifunctional peptide located in the N-terminal region at amino acids 17–41 of the lactoferrin protein, is characterized by a disulfide bond, an abundance of basic and hydrophobic amino acids, and a net positive charge of 8+ [1]. Lactoferricin exhibits multiple biological activities, including bacteriostatic, antiviral, antiprotozoal, tumor-suppressive, and cell apoptosis induction properties [1,7].

Recombinant peptide expression in microbial cells has attracted widespread attention due to its low cost and simple preparation process [3,4]. For example, the pPIC9K-His plasmid has been used to express the bovine lactoferricin-derived peptide in *Pichia pastoris* GS115, with methanol as the inducer [4]. Moreover, Lee et al. used the promoter P<sub>trmQ</sub> to express six tandem repeats of lactoferricin in *Bacillus subtilis* [8]. However, the levels of lactoferricin produced via recombinant expression in microbial cells are relatively low, and further research is needed

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to develop more efficient production methods.

*P. pastoris* has clear advantages in the protein processing, extracellular secretion, post-translational modifications, and glycosylation, which is now widely used for the expression of exogenous proteins or peptides [9,10]. In this study, we first performed host cell selection and signal peptide (SP) screening to express and extracellularly secrete the lactoferricin peptide in *P. pastoris*. Then, the fusion of lactoferricin with anionic antioxidant peptides alleviated its positive charge and enhanced lactoferricin synthesis, achieving a titer of 55.3 mg/L. The antibacterial activity of the recombinant lactoferricin peptide against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* was assessed using the Oxford cup method. Our results demonstrated that recombinant lactoferricin exhibited a potent antibacterial activity, suggesting that our approach has potential for industrial production of antimicrobial peptides.

## 2. Materials and methods

### 2.1. Chemicals and reagents

Unless otherwise specified, all chemicals and the bicinchoninic acid (BCA) Protein Assay Kit were purchased from Sangon Biotech Co., Ltd. (Shanghai, China). The plasmid extraction and DNA gel purification kits were purchased from Thermo Scientific (Waltham, USA). The Gibson Assembly Kit was purchased from New England Biolabs (Beijing, China). The primers used in this study were synthesized by GENEWIZ (Suzhou, China).

### 2.2. Plasmids and strains

The strains and plasmids used in this study are listed in Table 2, while the primers are listed in Table S1. The *E. coli* DH5 $\alpha$  strain was used for plasmid construction, and *P. pastoris* GS115, X-33, SMD1163, and SMD1168 strains were used as the host cells for recombinant expression of the lactoferricin. *P. pastoris* was transformed via electrotransformation [11].

### 2.3. Cultivation conditions

Luria–Bertani (LB) medium (10 g/L tryptone, 10 g/L NaCl, 5 g/L yeast extract) was used to culture *E. coli*, and yeast extract peptone dextrose (YPD) medium (20 g/L tryptone, 20 g/L glucose, 10 g/L yeast extract) was used to culture *P. pastoris*. Ampicillin (100  $\mu$ g/mL) was used to screen *E. coli* transformants, while zeocin (100  $\mu$ g/mL) were used as selection reagents of *P. pastoris* transformants.

Shake flask fermentation was performed using BMGY (Buffered Glycerol-complex Medium) medium (20 g/L peptone, 10 g/L yeast extract, 0.1 M potassium phosphate buffer [pH 6.0], 1.34% yeast nitrogen base,  $4 \times 10^{-5}$ % biotin, and 1% glycerin). BMMY (Buffered Methanol-complex Medium) medium can be produced by replacing 1% glycerin in BMGY medium with 0.5%–1% methanol. BSM medium (26.7 ml/L 85% phosphoric acid, 40.0 g/L glycerin, 14.9 g/L magnesium sulfate-7H<sub>2</sub>O, 18.2 g/L potassium sulfate, 4.13 g/L potassium hydroxide, and 0.93 g/L calcium sulfate) was used for fed-batch culture. PTM1 consisted of 65.0 g/L ferrous sulfate-7H<sub>2</sub>O, 20.0 g/L zinc chloride, 6.0 g/L copper sulfate-5H<sub>2</sub>O, 3.0 g/L manganese sulfate-H<sub>2</sub>O, 0.08 g/L sodium iodide, 0.5 g/L cobalt chloride, 0.2 g/L biotin, 0.2 g/L sodium molybdate-2H<sub>2</sub>O, 0.02 g/L boric acid, and 5.0 ml/L sulfuric acid.

The single colonies of *P. pastoris* were cultured using YPD medium at 30 °C and 220 rpm in a shaken test tube for 16 h. The seed culture bacteria were further inoculated into a 500 mL baffled flask containing 50 mL BMMY medium at a 10% ratio and incubated for 36 h. Subsequently, the cells were harvested, washed, and inoculated into BMGY medium at an equivalent volume. The medium was supplemented daily with 1% methanol. Cell suspension samples were collected every 24 h to measure optical density at 600 nm (OD<sub>600</sub>) and to perform SDS-PAGE analysis.

For fed-batch culture, the seed culture was performed in baffled 500-mL shake flasks containing 150 mL YPD medium and then incubated for 16 h. The seed culture was inoculated into a 3-L fermenter (T&J Bio-engineering Co., Ltd., Shanghai, China) containing 1 L of fermentation medium. The pH was maintained at 5.5 by adding NH<sub>3</sub> H<sub>2</sub>O. A total of 12 ml/L PTM1 was added to the additional glycerol and methanol, and the methanol concentration was adjusted based on dissolved oxygen levels.

### 2.4. Antibacterial activity assay

*E. coli*, *B. subtilis*, and *S. aureus* colonies were inoculated into tubes containing LB medium, and cells were grown for 16 h at 37 °C and 220 rpm. The antibacterial activity of the samples was analyzed using the agar diffusion method. Petri dishes were coated with LB agar medium and 100  $\mu$ L of 16 h culture of the indicator strain. After punching 6-mm-diameter wells into agar, 100  $\mu$ L of the culture supernatant was loaded into each well, and the plates were incubated at 37 °C for 16 h.

### 2.5. SDS-PAGE and western blotting

After 24 h of fermentation, 1 mL of each of the culture samples was collected and used to perform centrifugation at 12,000 $\times$ g and 4 °C for 8 min. The resulting fermentation broth supernatant was treated with 4 $\times$  sample buffer and boiled for 10 min. The prepared samples and markers were separated via SDS-PAGE on a 10% Bolt Bis-Tris Plus gel using MES SDS running buffer (Thermo Scientific, Waltham, USA). Subsequently, the proteins with different molecular weights were visualized via Coomassie Brilliant Blue R250 staining.

For Western blot analysis, the separated recombinant proteins were transferred from gels to 0.2- $\mu$ m polyvinylidene difluoride membranes. The membranes were blocked for 1 h in QuickBlock™ Blocking Buffer (Beyotime, Shanghai, China), and then incubated overnight at 4 °C with primary mouse monoclonal antibody against His tag (1:1000 dilution). After washing three times in Tris-Buffered Saline Tween-20, the membranes were incubated with a goat antimouse secondary antibody (1:1000 dilution). The results of the Western blot analysis were visualized via color-developing using the ECL Color Development Kit.

### 2.6. Lactoferricin purification

The supernatant of the fermentation broth was collected via centrifugation at 6000 $\times$ g and then purified using the Ni Beads 6FF Gravity column (Smart-Lifesciences). First, the column was washed with 10 column volumes of phosphate-buffered saline (PBS), and the supernatant was loaded onto the pre-balanced gravity column. Next, 10 column volumes of washing buffer with 5 mM imidazole in PBS were added to wash the column. Subsequently, the His-tagged proteins were eluted using 4 column volumes of an elution buffer with 250 mM imidazole in PBS, collecting fractions with each column volume in individual tubes. Afterward, the purity of the lactoferricin was assessed using SDS-PAGE. If only a single target band was observed (Fig. S1), quantification of the purified lactoferricin was performed using BCA Protein Assay Kit.

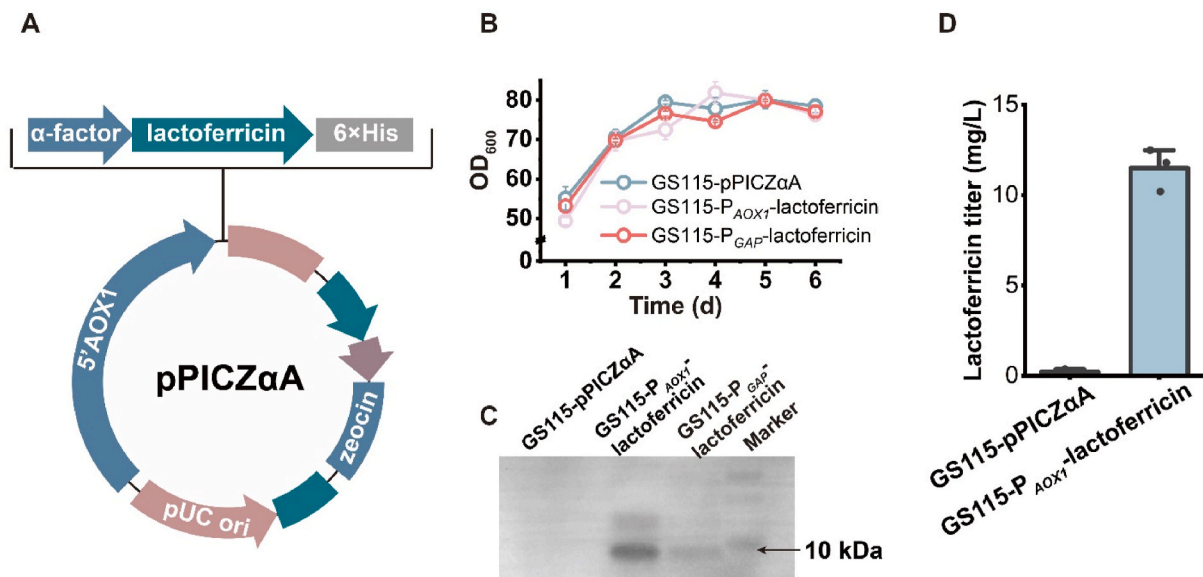
### 2.7. Statistical analysis

All experiments were independently repeated at least three times, and the results were expressed as mean  $\pm$  standard deviation. Statistical comparisons were performed using the two-sided *t*-test in Excel. *P* values of <0.05 were considered statistically significant.

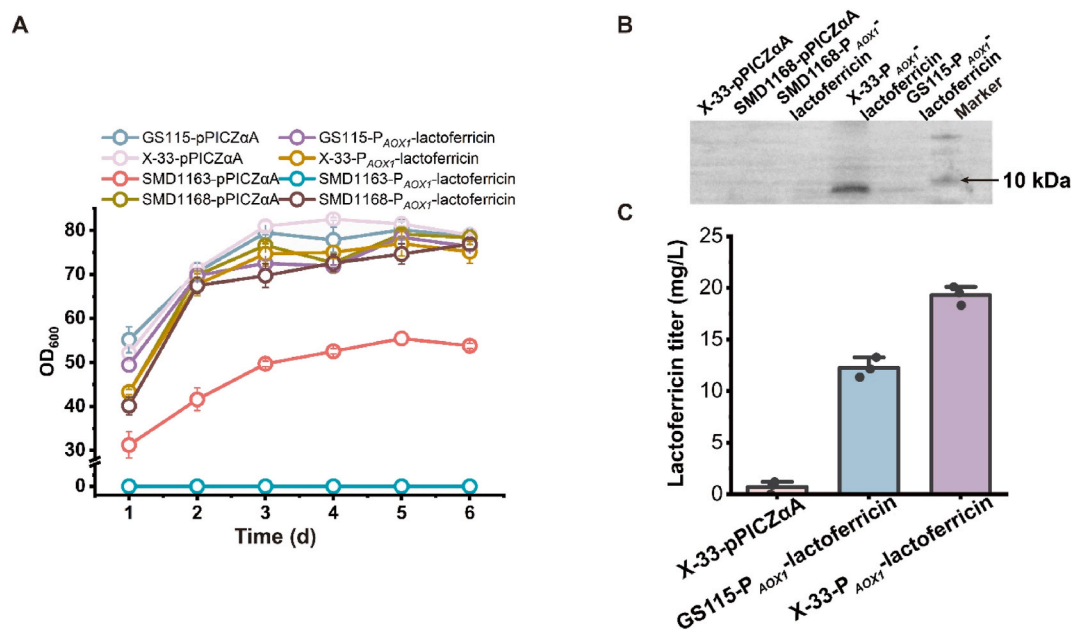
## 3. Results and discussion

### 3.1. Recombinant expression and host selection of lactoferricin

The recombinant expression of the lactoferricin was achieved by



**Fig. 1.** Lactoferrin expression in *Pichia pastoris*. (A) Structure of the recombinant plasmid. (B) Comparison between growth curves of GS115- $P_{AOX1}$ -lactoferrin and GS115- $P_{GAP}$ -lactoferrin strains and the GS115-pPICZ $\alpha$ A strain in the baffled flask. (C) Western blot analysis was performed to detect the expression of lactoferrin. (D) The production of GS115- $P_{AOX1}$ -lactoferrin was detected via the bicinchoninic acid (BCA) protein assay.



**Fig. 2.** Comparative analysis of lactoferrin expression profiles in different strains. (A) Comparison of the growth curve among different strains. (B) Western blot analysis was conducted to detect the effects of different genotypes on lactoferrin synthesis. (C) The BCA protein assay was used to detect the synthesis of lactoferrin in the X-33- $P_{AOX1}$ -lactoferrin strain.

inserting a 75-bp DNA fragment encoding the codon-optimized lactoferrin into the plasmid pPICZ $\alpha$ A containing the inducible promoter  $P_{AOX1}$ ,  $\alpha$ -factor secretory SP and 6-His tag (Fig. 1A). The inducible promoter  $P_{AOX1}$  was then replaced by the constitutive promoter  $P_{GAP}$  to compare the relative levels of lactoferrin under inducible and constitutive conditions, respectively. The recombinant plasmids were transformed into *P. pastoris* GS115 to generate recombinant GS115- $P_{AOX1}$ -lactoferrin and GS115- $P_{GAP}$ -lactoferrin strains. The cell density of GS115- $P_{AOX1}$ -lactoferrin and GS115- $P_{GAP}$ -lactoferrin strains, as measured at OD<sub>600</sub>, did not show significant changes compared to the control strains expressing the pPICZ $\alpha$ A plasmid (Fig. 1B), suggesting that low doses of lactoferrin peptide did not inhibit the growth of

*P. pastoris*. After 144 h of fermentation in shaking flasks, the supernatants of the fermentation broth were harvested for Western blot analysis. As shown in Fig. 1C, a weak band was detected in the supernatant of the GS115- $P_{GAP}$ -lactoferrin strain, and the band in the supernatant of the GS115- $P_{AOX1}$ -lactoferrin strain was much stronger than that of the GS115- $P_{GAP}$ -lactoferrin strain, with a titer of 11.5 mg/L (Fig. 1C and D). These results indicate that lactoferrin was successfully expressed and secreted using the inducible  $P_{AOX1}$  promoter. Therefore,  $P_{AOX1}$  was selected to carry out the following experiments.

The ability of different genotypes to express exogenous proteins can be highly variable [12,13]. Therefore, we examined the expression of lactoferrin in four *P. pastoris* strains, including GS115, X-33, SMD1163,

**Table 1**  
The sequences and source of signal peptides.

signal peptides	Amino acid sequences	Source
0030	MKFAISTLLIILQAAAVFAAF	PAS_chr3_0030 signal peptide from <i>P. pastoris</i> .
INU1	MKLAYSLLLPLAGVSASVINYKR	Inulinase signal peptide from <i>Kluyveromyces marxianus</i> .
MEL1	MRAFLFLTACISLPGVFGVNET	$\alpha$ -galactosidase signal peptide from <i>Saccharomyces cerevisiae</i> .
OST1	MRQVWFWSWIVGLFLCFNVSSA	Ost1 signal peptide from <i>S. cerevisiae</i> .
PHO11	MLKSAVYSILAASLVNA	Acid phosphatase signal peptide from <i>S. cerevisiae</i> .
SCW10	MQVKSIVNLLLACSLAVA	SCW10 signal peptide from <i>P. pastoris</i>
SP	MKLVFLVLLFLGALGLCLA	Lactoferrin signal peptide from <i>Bos taurus</i>
SUC2	MLLQAFLLLAGFAAKISA	Invertase signal peptide from <i>S. cerevisiae</i> .
UTH1	MKSQLIFMALASLVAS	UTH1 signal peptide from <i>K. marxianus</i> .

and SMD1168. The recombinant pPICZ $\alpha$ A-lactoferrin plasmid was transferred into X-33, SMD1163, and SMD1168 strains to obtain X33- $P_{AOXI}$ -lactoferrin, SMD1163- $P_{AOXI}$ -lactoferrin, and SMD1168- $P_{AOXI}$ -lactoferrin strains, respectively. As shown in Fig. 2A, except for the SMD1163- $P_{AOXI}$ -lactoferrin strain, which hardly grew, the OD<sub>600</sub> of the X33- $P_{AOXI}$ -lactoferrin, SMD1168- $P_{AOXI}$ -lactoferrin, and GS115- $P_{AOXI}$ -lactoferrin strains did not show significant changes compared to the control strains. The Western blot analysis revealed that the lactoferrin level in the X33-lactoferrin strain was significantly higher than that in SMD1168- $P_{AOXI}$ -lactoferrin and GS115- $P_{AOXI}$ -lactoferrin strains (Fig. 2B). The BCA analysis indicated that the X33- $P_{AOXI}$ -lactoferrin strain produced 19.3 mg/L of lactoferrin, which is 1.7 fold more than the titer produced by the GS115- $P_{AOXI}$ -lactoferrin strain (11.5 mg/L) (Fig. 2C). The above results demonstrate that, for lactoferrin expression, the X-33 strain is a host with a significant advantage. The speculated main reason is that the wild-type X-33 strain has better resistance, such as its enhanced ability to resist metabolic stress or toxicity caused by the overexpression of proteins or peptides. Additionally, similar conclusions were drawn in the expression of HIV-negative factor and human interleukin-1 beta proteins [12,13]. Therefore, the X33-lactoferrin strain was selected for further studies.

### 3.2. SP screening for lactoferrin secretion

SPs are short, N-terminal peptides ubiquitously present in eukaryotic and prokaryotic cells, which play a role in marking the protein for secretion and target to specific cellular locations [14]. We screened out the most suitable SP for lactoferrin by comparing the secretion of lactoferrin with different SPs, including 0030, INU1, MEL1, OST, PHO11, SCW10, SP, SUC2, and UTH1 (Table 1). The  $\alpha$ -factor secretory SP of the pPICZ $\alpha$ A-lactoferrin plasmid was sequentially replaced with these SPs to generate 0030-lactoferrin, INU1-lactoferrin, MEL1-lactoferrin, OST-lactoferrin, PHO11-lactoferrin, SCW10-lactoferrin, SP-lactoferrin, SUC2-lactoferrin, and UTH1-lactoferrin strains. As shown in Fig. 3A, the production of lactoferrin was significantly reduced when the nine SPs were used to replace the  $\alpha$ -factor secretory SP, and no immunoblot band was observed.

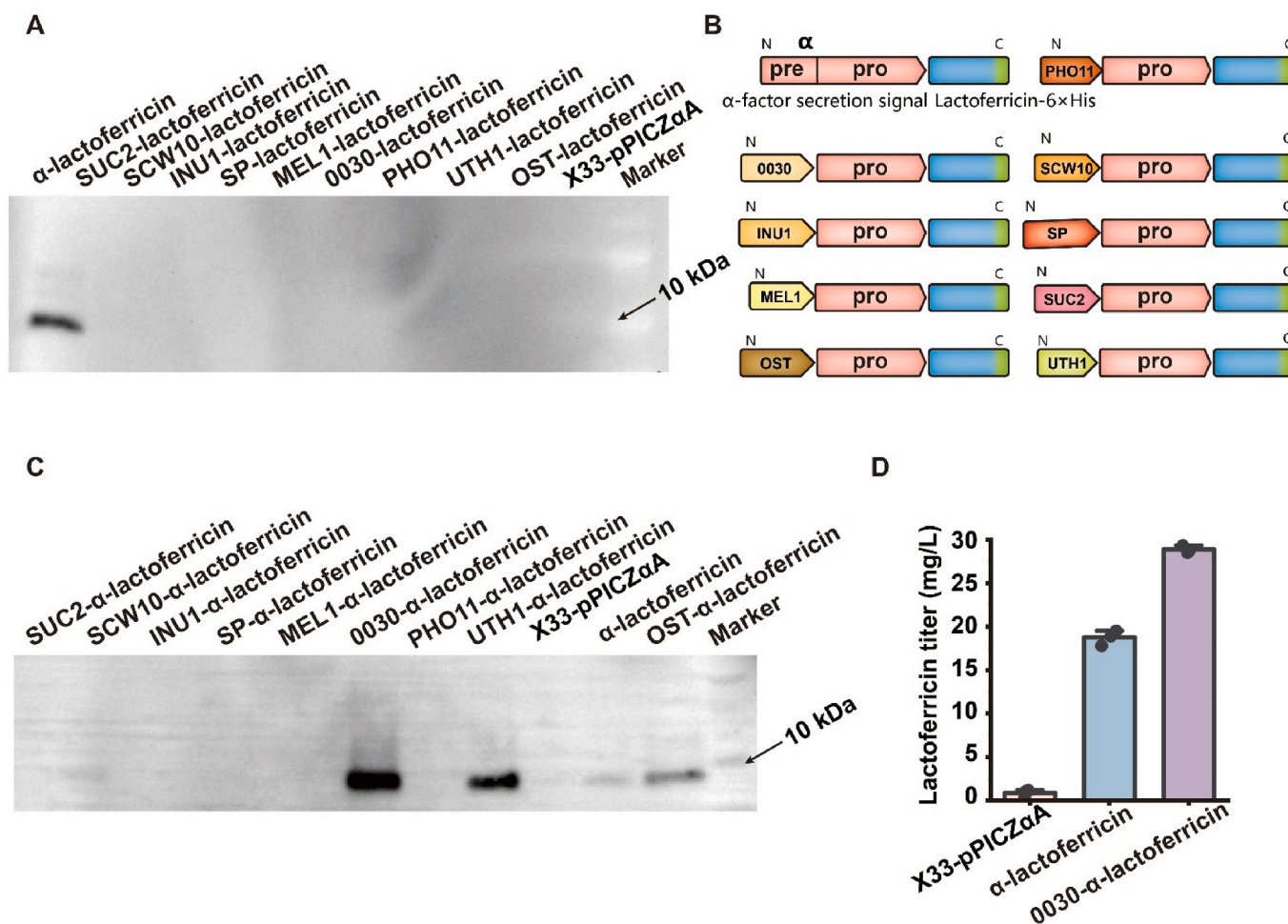
Conventional SPs consist solely of the pre sequence, while the  $\alpha$ -factor secretory SP is comprised of both pre- and pro-sequences, with the latter containing an endoplasmic reticulum (ER) export signal that is specifically recognized by the transmembrane Erv29 receptor [15]. The receptor-mediated export likely contributes to the efficacy of the  $\alpha$ -factor secretory SP in facilitating heterologous protein production [15]. But a limitation of the  $\alpha$ -factor secretory SP is that the signal sequence portion directs posttranslational translocation across the ER

**Table 2**  
Strains and plasmids used in this study.

Strains/Plasmids	Characteristic	Source <sup>a</sup>
<b>Strains</b>		
<i>E. Coli</i> DH5 $\alpha$	F <sup>-</sup> $\phi$ 80, <i>lacZ</i> $\Delta$ M15, $\Delta$ ( <i>lacZYA-argF</i> )U169, <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (rk-, mk+), <i>phoA</i> , <i>supE</i> 44, <i>thi-1</i> , <i>gyrA</i> 96, <i>relA1</i> , $\lambda$	Lab stock
<i>P. pastoris</i> SMD1163	his4 pep4 prb 1	Lab stock
<i>P. pastoris</i> SMD1168	his4, pep4	Lab stock
<i>P. pastoris</i> GS115	Wild type	Lab stock
<i>P. pastoris</i> X-33	Wild type	Lab stock
GS115- $P_{AOXI}$ -lactoferrin	GS115 strain with pPICZ $\alpha$ A-lactoferrin	This study
GS115- $P_{GAP}$ -lactoferrin	GS115 strain with pGAPZ $\alpha$ A-lactoferrin	This study
X33- $P_{AOXI}$ -lactoferrin	X-33 strain with pPICZ $\alpha$ A-lactoferrin	This study
SMD1163- $P_{AOXI}$ -lactoferrin	SMD1163 strain with pPICZ $\alpha$ A-lactoferrin	This study
SMD1168- $P_{AOXI}$ -lactoferrin	SMD1168 strain with pPICZ $\alpha$ A-lactoferrin	This study
0030-lactoferrin	X-33 strain with pPICZ $\alpha$ A-0030-lactoferrin	This study
INU1-lactoferrin	X-33 strain with pPICZ $\alpha$ A-INU1-lactoferrin	This study
MEL1-lactoferrin	X-33 strain with pPICZ $\alpha$ A-MEL1-lactoferrin	This study
OST-lactoferrin	X-33 strain with pPICZ $\alpha$ A-OST-lactoferrin	This study
PHO11-lactoferrin	X-33 strain with pPICZ $\alpha$ A-PHO11-lactoferrin	This study
SCW10-lactoferrin	X-33 strain with pPICZ $\alpha$ A-SCW10-lactoferrin	This study
SP-lactoferrin	X-33 strain with pPICZ $\alpha$ A-SP-lactoferrin	This study
SUC2-lactoferrin	X-33 strain with pPICZ $\alpha$ A-SUC2-lactoferrin	This study
UTH1-lactoferrin	X-33 strain with pPICZ $\alpha$ A-UTH1-lactoferrin	This study
0030- $\alpha$ -lactoferrin	X-33 strain with pPICZ $\alpha$ A-0030- $\alpha$ -lactoferrin	This study
INU1- $\alpha$ -lactoferrin	X-33 strain with pPICZ $\alpha$ A-INU1- $\alpha$ -lactoferrin	This study
MEL1- $\alpha$ -lactoferrin	X-33 strain with pPICZ $\alpha$ A-MEL1- $\alpha$ -lactoferrin	This study
OST- $\alpha$ -lactoferrin	X-33 strain with pPICZ $\alpha$ A-OST- $\alpha$ -lactoferrin	This study
PHO11- $\alpha$ -lactoferrin	X-33 strain with pPICZ $\alpha$ A-PHO11- $\alpha$ -lactoferrin	This study
SCW10- $\alpha$ -lactoferrin	X-33 strain with pPICZ $\alpha$ A-SCW10- $\alpha$ -lactoferrin	This study
SP- $\alpha$ -lactoferrin	X-33 strain with pPICZ $\alpha$ A-SP- $\alpha$ -lactoferrin	This study
SUC2- $\alpha$ -lactoferrin	X-33 strain with pPICZ $\alpha$ A-SUC2- $\alpha$ -lactoferrin	This study
UTH1- $\alpha$ -lactoferrin	X-33 strain with pPICZ $\alpha$ A-UTH1- $\alpha$ -lactoferrin	This study
PEP1-lactoferrin	X-33 strain with pPICZ $\alpha$ A-0030- $\alpha$ -PEP1-lactoferrin	This study
PEP2-lactoferrin	X-33 strain with pPICZ $\alpha$ A-0030- $\alpha$ -PEP2-lactoferrin	This study
PEP1/2-lactoferrin	X-33 strain with pPICZ $\alpha$ A-0030- $\alpha$ -PEP1/2-lactoferrin	This study
<b>Plasmids</b>		
pPICZ $\alpha$ A	$P_{AOXI}$ promoter, $\alpha$ -factor secretory signal peptide, 6-His tag, Zeocin	Lab stock
pGAPZ $\alpha$ A	$P_{GAP}$ promoter, $\alpha$ -factor secretory signal peptide, 6-His tag, Zeocin	Lab stock

membrane, as a result, if the  $\alpha$ -factor secretory SP is fused to a protein that can fold in the yeast cytosol, the protein may be unable to cross the ER membrane to initiate the secretory pathway. Replacing the pre-sequence of  $\alpha$ -factor secretory SP with the Ost1 SP could increase





**Fig. 3.** Signal peptide screening for lactoferrin secretion. (A) Western blot analysis was performed to detect the effects of different signal peptides on lactoferrin synthesis. (B) The schematic diagram of hybridization of different signal peptides. (C) The Western blot analysis was used to detect the effects of hybridized signal peptides on lactoferrin synthesis. (D) The BCA protein assay was used to compare the production of lactoferrin in  $\alpha$ -lactoferrin and 0030- $\alpha$ -lactoferrin strains.

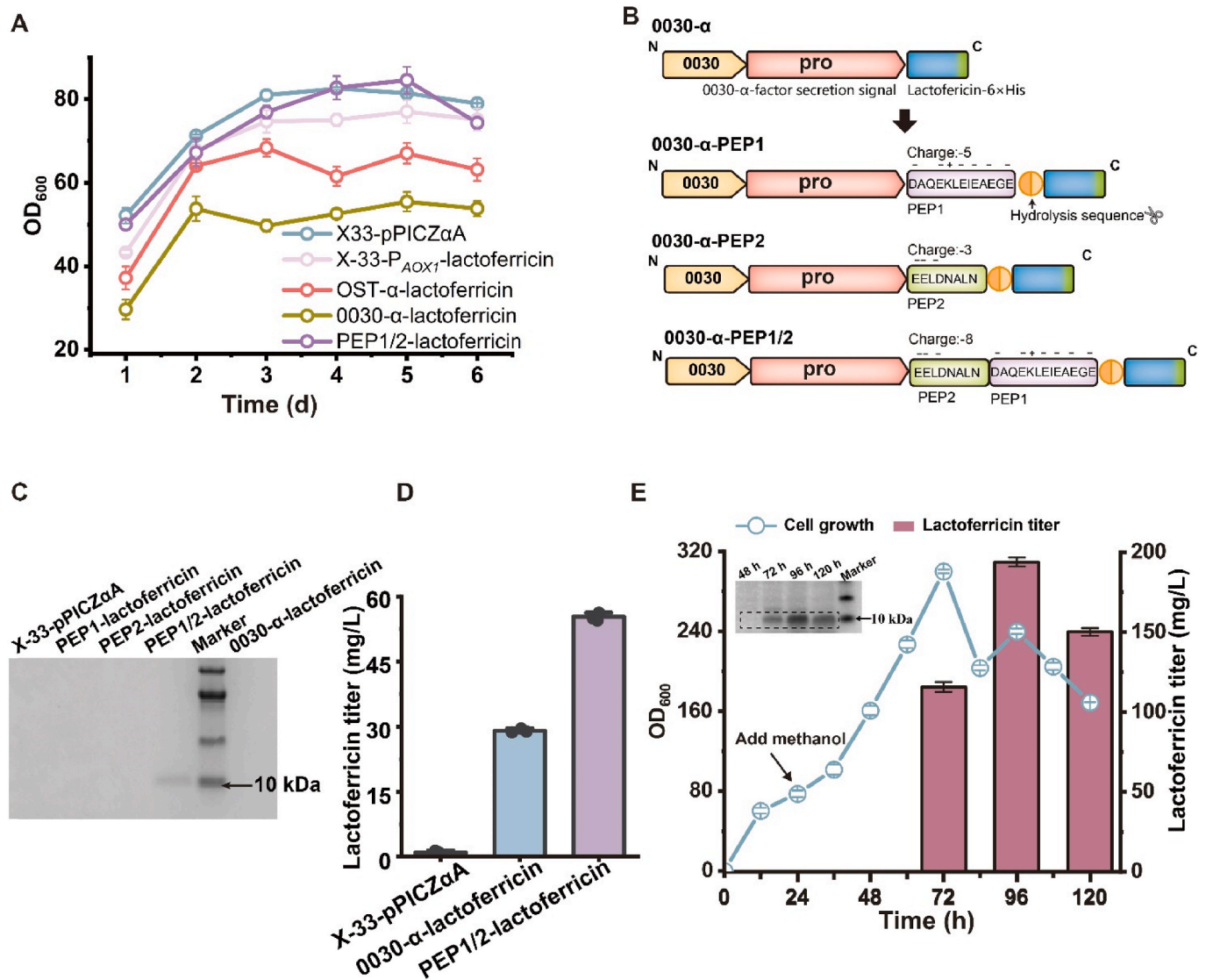
protein expression [16]. For example, the secretion levels of E2-Crimson and lipase BTL2 were significantly increased 20 and 10-fold compared to the original  $\alpha$ -factor secretion SP, respectively, when a hybrid SP consisting of OST1 and the  $\alpha$ -factor pro-sequence was used [16]. We attempted to hybridize the  $\alpha$ -factor secretory SP by replacing its pre-sequence with that of 0030, INU1, MEL1, OST, PHO11, SCW10, SP, SUC2, and UTH1 SPs (Fig. 3B). The resulting strains were named as follows: 0030- $\alpha$ -lactoferrin, INU1- $\alpha$ -lactoferrin, MEL1- $\alpha$ -lactoferrin, OST- $\alpha$ -lactoferrin, PHO11- $\alpha$ -lactoferrin, SCW10- $\alpha$ -lactoferrin, SP- $\alpha$ -lactoferrin, SUC2- $\alpha$ -lactoferrin, and UTH1- $\alpha$ -lactoferrin. The titers of lactoferrin in 0030- $\alpha$ -lactoferrin, OST- $\alpha$ -lactoferrin, UTH1- $\alpha$ -lactoferrin strains were significantly higher than that in the X33- $P_{AOX1}$ -lactoferrin strain, with the highest titer observed in the 0030- $\alpha$ -lactoferrin strain (28.8 mg/L) (Fig. 3C and D). These results suggest that hybrid SPs can combine the properties of different SPs to enhance the secretion of recombinant proteins or peptides.

### 3.3. Fusion of anion antioxidant peptides promotes lactoferrin synthesis

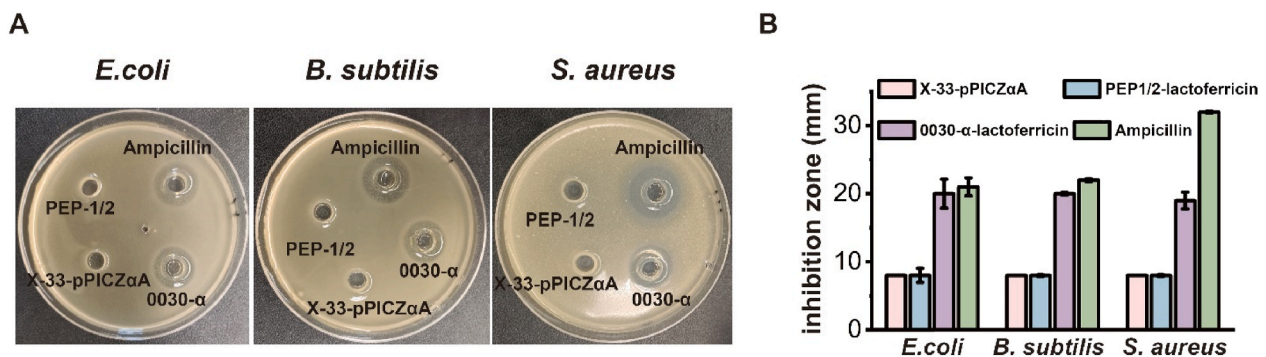
Our experimental results show that the increase in lactoferrin synthesis was correlated with a decrease in  $OD_{600}$  (Fig. 4A), suggesting that high doses, especially those exceeding 20 mg/L, lactoferrin has an inhibitory effect on *P. pastoris*. It has been shown that the antibacterial activity of antimicrobial peptides is positively correlated with the number of net positive charges [1]. For example, the net positive charge of bovine lactoferrampin (5+) has been associated with higher

antibacterial activity than that of human lactoferrampin, which has a reduced positive charge (2+) [17]. Indeed, the antibacterial activity of human lactoferrampin can be significantly improved by artificially increasing its net positive charge [18]. Because the net positive charge of lactoferrin is 8+ [1], we attempted to alleviate the antimicrobial effect by fusing lactoferrin with anionic antioxidant peptides, thereby neutralizing the positive charges.

The anionic antioxidant peptides PEP1 and PEP2, which consist of DAQEKLEIEAEGE and EELDNALN amino acid sequences, respectively, were selected from the porcine myofibrillar protein. PEP1 and PEP2 peptides have five and three net negative charges, respectively [3]. Single PEP1 and PEP2 peptides and the tandem PEP1-PEP2 dipeptide were fused at the N-terminal region of lactoferrin (Fig. 4B), and the resulting strains were named PEP1-lactoferrin, PEP2-lactoferrin, and PEP1/2-lactoferrin, respectively. SDS-PAGE analysis after 6 days of fermentation showed that the PEP1/2-lactoferrin strain exhibited the highest titer (55.3 mg/L). These results suggest that neutralizing the positive charge may enhance lactoferrin expression (Fig. 4C and D). The lactoferrin synthesis ability of the PEP1/2-lactoferrin was further validated in a 3-L bioreactor. The titer of lactoferrin produced by the PEP1/2-lactoferrin was monitored at 24 h intervals following the addition of methanol. The BCA protein assay revealed that after 96 h of fermentation, the titer of lactoferrin reached a maximum of 193.9 mg/L. With the extension of fermentation time, lactoferrin underwent partial degradation, and by 120 h of fermentation, the titer decreased to 150.3 mg/L (Fig. 4E). To the best of our knowledge, this is the highest



**Fig. 4.** The fusion of anion antioxidant peptides promotes the synthesis of lactoferricin. (A) Effects of lactoferricin expression on strain growth. (B) Structure of lactoferricin fused with anionic antioxidant peptides. Anionic antioxidant peptides were ligated to the N-terminus of the gene via hydrolysis. (C) SDS-PAGE was used to detect the effects of antioxidant peptides on the synthesis of lactoferricin. (D) A BCA protein assay was performed to compare lactoferricin production in 0030-α-lactoferricin and PEP1/2-lactoferricin strains. (E) Growth curve and lactoferricin production of the PEP1/2-lactoferricin strain during fermentation, with methanol added after 24 h of culture.



**Fig. 5.** Antimicrobial activity of recombinant-expressed lactoferricin. (A) The inhibition zones of the 0030-α-lactoferricin strain and PEP1/2-lactoferricin strain against *Escherichia coli*, *Staphylococcus aureus*, and *Bacillus subtilis* are shown. References: 0030-α: sixth-day culture supernatant of the 0030-α-lactoferricin strain. PEP1/2: sixth-day culture supernatant of the PEP1/2-lactoferricin strain. Ampicillin: 100 μg/mL, positive control. X-33-pPICZαA: sixth-day culture supernatant of the X-33-pPICZαA strain, negative control. (B) The inhibition zones formed by the culture supernatants of the 0030-α-lactoferricin strain, PEP1/2-lactoferricin strain, the positive control, and the negative control were measured and compared. The diameter of the Oxford cup hole was 8 mm.

titer of lactoferricin produced by recombinant expression.

### 3.4. Antimicrobial activity of recombinant-expressed lactoferricin

The antimicrobial activity of recombinant lactoferricin was assessed in *E. coli*, *B. subtilis*, and *S. aureus* test strains. The fermentation supernatants of the strains with the empty pPICZαA plasmid and 100 µg/L of ampicillin were used as negative and positive controls, respectively. As shown in Fig. 5A and B, the negative control did not exhibit antibacterial activity. The 0030-α-lactoferricin strain showed potent antibacterial activity against *E. coli*, *B. subtilis*, and *S. aureus*, as evidenced by the diameters of the inhibition zone (20, 20, and 19 mm, respectively). In contrast to 0030-α-lactoferricin, the antibacterial activity of the PEP1/2-lactoferricin strain was significantly weak, as evidenced by the almost complete absence of an inhibition zone (Fig. 5A and B). The lower antibacterial activity of PEP1/2-lactoferricin may be attributed to the decrease in its positive charge, which is consistent with previous reports [1]. These results suggest that the net positive charge of lactoferricin plays a role in its antimicrobial activity, which is supported by the observation that recombinant lactoferricin exhibits significantly higher antibacterial activity than fusion peptides.

## 4. Conclusions

In this study, host cell selection, SP screening, and fusion expression of anionic antioxidant peptides enabled efficient recombinant expression of lactoferricin to maximum titers of 55.3 mg/L and 193.9 mg/L in shaking flask and a 3-L bioreactor, respectively. The agar diffusion assay showed that recombinant lactoferricin exhibited potent antimicrobial activity against gram-negative and gram-positive bacteria, indicating that it is an efficient broad-spectrum AMP. The findings of this study provide valuable information for the development of cell factories for AMPs.

### CRedit authorship contribution statement

**Xueqin Lv:** Conceptualization, Investigation, Methodology, Visualization, Writing – original draft. **Yuting Zhang:** Investigation, Methodology. **Lingrui Wang:** Methodology. **Shixiu Cui:** Investigation. **Yanfeng Liu:** Writing – review & editing. **Jianghua Li:** Supervision. **Guocheng Du:** Supervision, Resources. **Long Liu:** Conceptualization, Supervision, Funding acquisition, Resources, Writing – review & editing.

### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.synbio.2023.12.002>.

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