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Recombinant of the Staphylococcal Bacteriophage Lysin CHAP$_k$ and Its Elimination against *Streptococcus agalactiae* Biofilms

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Abstract: Bovine mastitis is the most important infectious disease, causing significant losses in the dairy industry, in which *Streptococcus agalactiae* is a major pathogen. In this study, lysin CHAP$_k$, derived from bacteriophage K, was expressed heterogeneously, and its antimicrobial and anti-biofilm effects against *S. agalactiae* isolated from bovine mastitis were further analyzed. CHAP$_k$ was expressed in *Escherichia coli* BL21 (DE3), in which the purified yield of CHAP$_k$ was up to 14.6 mg/L with the purity of 95%. Time-killing kinetic curves showed that CHAP$_k$ fastly killed *S. agalactiae* in TSB medium and in milk within 25 min (by 3.3 $\log_{10}$ CFU/mL and 2.4 $\log_{10}$ CFU/mL, respectively). Observation of scanning electron microscope (SEM) showed cells wrinkled and ruptured after the treatment of CHAP$_k$. CHAP$_k$ effectively inhibited early biofilms by 95% in 8 × MIC, and eradicated mature biofilms by 89.4% in 16 × MIC. Moreover, CHAP$_k$ killed 99% bacteria in mature biofilms. Confocal laser scanning microscopy (CLSM) also demonstrated the potent antimicrobial and anti-biofilm action of CHAP$_k$. It was firstly demonstrated CHAP$_k$ had the characters of inhibition/elimination of *S. agalactiae* biofilms and killing the bacteria in biofilms. CHAP$_k$ has the potential to develop a new antibacterial agent for mastitis treatment of *S. agalactiae* infections.

Keywords: Lysin; CHAP$_k$; expression; anti-biofilm ability; *Streptococcus agalactiae*

1. Introduction

Streptococcus agalactiae of group B (GBS) is one of the important pathogens which is associated with clinical/subclinical mastitis in bovine and neonatal sepsis and meningitis in humans. Bovine mastitis is the primary health hazard, leading to severe reduction of milk production and effect of milk quality. It is responsible for significant economic losses in the dairy industry worldwide [1,2]. Infection control measures of *S. agalactiae* were performed during the 1960s to reduce the occurrence of *S. agalactiae* mastitis in several European countries. However, the prevalence of *S. agalactiae* mastitis in bovine has re-emergence in Norway, Denmark, and other countries [3–5]. In China, *S. agalactiae* is also a prevalent problem in bovine diagnosed with subclinical mastitis [6]. The sequence types (STs) of *S. agalactiae* in bovine mainly belong to ST67, ST103, and ST568, which harbor the virulence characteristics of biofilms formation ability, growth ability in milk and adhesion ability, and can persist indefinitely within the mammary gland [1,7]. β-lactam antibiotics (BLAs) (penicillin, ampicillin, cefalexin, and ceftiofur

sodium) are officially approved and extensively used to treat *S. agalactiae* mastitis. However, clinical isolated *S. agalactiae* from cows with mastitis showed unpredicted mutations in the penicillin-binding proteins encoding (PBP) genes which lead to the high BLAs-resistant rate [8]. Eight *S. agalactiae* strains isolated from cow mastitis showed 37.5% resistant to tetracycline and were highly resistant to trimethoprim [9]. Therefore, there is an urgent need to search for novel agents against *S. agalactiae* with low possibility to develop resistance.

Lysins are peptidoglycan hydrolase produced by bacteriophages. Among the different sources of bacteriophages, lysins show specificity on the pathogen. Compared with phages, non-biological lysins are safer and have no effect on commensal microflora [10,11]. Previous studies have demonstrated that lysins were active against streptococcus and staphylococcus pathogens, causing mucosal and systemic infections [12–15]. Lysins rapidly induce bacterial lysis and death by hydrolysing covalent bonds, which are essential for cell wall integrity and viability, and consequently develop little resistance compared with antibiotics [16–18]. $CHAP_k$ (18.6 kDa), comprised solely of the lytic domain, is a truncated derivative of native lysin (LysK, 54 kDa) of *Staphylococcus aureus*, which belongs to cysteine-and histidine-dependent amidohydrolase/peptidase and specifically cleaves staphylococcal cell wall on the peptide bond between D-alanine and the first glycine in the pentaglycine cross bridge [19–21]. Previous studies have reported that $CHAP_k$ exhibited strong activity against *S. aureus* and effected on the *S. aureus* biofilms [22–25]. Interestingly, researchers discovered that $CHAP_k$ also had antibacterial activity against streptococcus, which may be related to the similar peptidoglycan cross-bridge of staphylococcal and streptococcal pathogens [19,26]. In this study, *$CHAP_k$* gene was synthesized and expressed in *E. coli* BL21 (DE3), and the efficacy of recombinant $CHAP_k$ against *S. agalactiae* biofilms was evaluated.

2. Materials and Methods

2.1. Strains, Plasmid, and Reagents

The tested strains of *Streptococcus agalactiae* CAU-FRI 1, *S. agalactiae* CAU-FRI 2, and *S. agalactiae* CAU-FRI 3 isolated from bovine mastitis were obtained from China Agricultural University, *S. agalactiae* CAU-FRI 4 isolated from tilapia was stored at our laboratory, *Streptococcus uberis* CAU-FRI 1, *S. uberis* CAU-FRI 2, *S. uberis* CAU-FRI 3, *Streptococcus dysgalactiae* CAU-FRI 1, *S. dysgalactiae* CAU-FRI 2, and *S. dysgalactiae* CAU-FRI 3 isolated from bovine mastitis were obtained from China Agricultural University, *S. agalactiae* ATCC 13813, *S. aureus* ATCC 43300, *Streptococcus epidermidis* ATCC 12228, *S. epidermidis* ATCC 35984, and *Escherichia coli* ATCC 25922 were purchased from the American Type Culture Collection; *S. aureus* CVCC 546, *Streptococcus pneumoniae* CVCC 3309, *S. pneumoniae* CVCC 2350, *Streptococcus suis* CVCC 606, *S. suis* CVCC 3928, and *Salmonella enteritidis* CVCC 3377 were purchased from the China Veterinary Culture Collection Center (Beijing, China); *S. aureus* KY, *S. aureus* KR and *S. aureus* FJ isolated from bovine with endometritis were stored at our laboratory; *Staphylococcus hyicus* NCTC 10350 was purchased from National Collection of Type Cultures; *S. pneumoniae* CGMCC 1.8747 and *Candida albicans* CGMCC 2.2411 were purchased from China General Microbiological Culture Collection Center. pET28a vector and *E. coli* BL21 (DE3) (Novagen, Beijing, China) were used for cloning and expression. DNA restriction enzymes and T4 DNA ligase were purchased from NEB (USA). The kits for plasmid extraction and DNA purification were purchased from TIANGEN (China). Other chemical reagents were analytical grade.

2.2. Expression and Purification of $CHAP_k$

The synthesized *$CHAP_k$* (The sequence was acquired from NCBI PDB:4CT3_D and synthesized by Sangon Biotech (Shanghai) Co., Ltd.) nucleotide sequence was optimized by reverse translate tool (http://bioinformatics.org//sms2/rev_trans.html) based on the preferential codon usage of *E. coli* (http://www.kazusa.or.jp/codon/) and amplified with primers of *$CHAP_k$*-F: 5′-CATGCCATGGCGAAAACCCAGGCGGAAA-3′, *$CHAP_k$*-R: 5′-CCGCTCGAGCTATTAGTGGT

GGTGGTG-3′. The DNA fragment contained the *Xho*I and *Nco*I restriction site, and $8 \times$ His-tag was added to the C-terminus. After confirmed by sequencing the polymerase chain reaction (PCR) fragment, the purified *CHAP$_k$* fragment digested with *Xho*I and *Nco*I and ligated into pET28a vectors which digested with the same restriction enzymes to generate the pET28a-CHAP$_k$ plasmid, and the plasmid was transformed into *E. coli* BL21 (DE3). The positive transforms were screened on luria broth (LB, 1% NaCl, 0.5% Yeast extract, 1% Tryptone) plates containing 50 μg/mL kanamycin and confirmed by PCR and DNA sequencing.

The recombinant CHAP$_k$ protein was expressed in *E. coli* BL21 (DE3) by IPTG induction [27]. In brief, 1% of the positive transformant cultured overnight was inoculated into LB medium containing 50 μg/mL kanamycin and incubated to OD600 nm of 0.4–0.6. CHAP$_k$ expression was induced by IPTG at 1.0 mM, 37 °C for 6 h. The pellets were harvested by centrifugation at 4722 g for 5 min and analyzed by 12% SDS-PAGE. Cells were lysed by sonicated for 15 min at 0 °C, then collected precipitation and dissolved it in the binding buffer (20 mM sodium phosphate, 500 mM NaCl, 5 mM imidazole, 8 M Urea), samples were loading into Ni^{2+}–Nitriloacetate (NTA) super flow resin column at a rate of 4 mL/min with elution buffer (20 mM sodium phosphate, 500 mM NaCl, 175 mM imidazole, 8 M Urea, pH 7.4). Renaturation of the CHAP$_k$ by TGE buffer (50 mM Tris, 50 mM NaCl, 0.5 mM Ethylenediaminetetraacetic acid disodium salt (EDTA-Na$_2$), 0.1% L-Arginine, 10% glycerol, 6 M–4 M–2 M–1 M Urea gradient dialysis until changed to distilled water) at 4 °C. To confirm the purified protein, the mixture of purified protein and matrix SA was detected by matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI-TOF MS) (New UltrafleXtreme, Bruker, Germany) in linear mode (at the Laboratory of Proteomics, Institute of Biophysics, Chinese Academy of Sciences). Purified protein was freeze-dried and stored in –20 °C [28].

2.3. Antibacterial Activity Assay

The minimal inhibitory concentrations (MICs) of CHAP$_k$ were measured by the broth microdilution method. Overnight cultures were inoculated to the tryptic soy broth medium (TSB, Qingdao Hope Bio-Technology Co., Ltd.) containing 3% (*w/v*) BSA, cultured to the mid-log phage. The bacterial suspension was then diluted to 1×10^5 CFU/mL. CHAP$_k$ was two-fold diluted to final concentration with 0.625–1280 μg/mL. Then CHAP$_k$ (10 μL) and bacterial suspension (90 μL) were added into 96-well plates (polystyrene, Beijing Huamei World Trade Technology Development Co., Ltd.). The plates were incubated at 37 °C for 16–18 h [29]. All assays were performed in triplicate. The MIC was defined as the lowest protein concentration at which there was no visible bacterial growth.

2.4. Time-Killing Kinetic Curves in TSB and Milk

After *S. agalactiae* ATCC 13813 inoculated into TSB containing 3% BSA at 37 °C and 250 rpm for 3 h, the mid-log phase bacteria were diluted to 5×10^8 CFU/mL. CHAP$_k$ was mixed with bacteria suspension to the final concentration of $16 \times$ MIC (1.72 μM) in TSB, $32 \times$ MIC (3.44 μM) in pasteurized whole-fat milk (6% fat, 2% carbohydrate, Beijing Sanyuan food Co., Ltd.). Meanwhile, the bacteria suspension without CHAP$_k$ as the blank control, and $16 \times$ MIC and $32 \times$ MIC of vancomycin as the positive control, respectively. Sample (100 μL) was taken at 1, 3, 5, 10, 15, 20, and 25 min for colony counting on the Tryptic Soy Agar (TSA, Qingdao Hope Bio-Technology Co., Ltd.).

2.5. Scanning Electron Microscope (SEM) Observation

The mid-log phase *S. agalactiae* cells were incubated with $8 \times$ MIC CHAP$_k$ for 2 h at 37 °C. After being washed with PBS for three times, *S. agalactiae* cells treated with CHAP$_k$ were fixed with 2.5% glutaric dialdehyde (0.01 M PBS) at 4 °C overnight. Fixation fluid was discarded, and cells were washed with deionized water three times to removed fixation fluid for 4, 5, and 6 min, respectively. Then the samples were covered on the coverslips and various concentrations of ethanol (50%, 70%, 85%, 95%, and 100% $\times$ 3 times) were dehydrated for 15 min. Finally, the samples were dried by CO_2, coated with platinum, and observed on a QUANTA200 SEM [30,31].

2.6. Ability of CHAP$_k$ against Biofilms and Bacteria of S. agalactiae

2.6.1. Biofilms Formation Assay

The ability of biofilms formation was evaluated by crystal violet staining, which was referenced as described by Tremblay et al. [32]. Briefly, *S. agalactiae* ATCC 13813 cells were inoculated to TSB medium containing 3% BSA, incubated until mid-log phase, and diluted to 1×10^8 CFU/mL by TSB medium. A 200 μL bacterial suspension was inoculated into 96-well plates. After incubation for 24 h at 37 °C, the medium was gently rinsed by PBS for three times, excess liquid was dried in room temperature with an inverted position, and the wells were fixed with 2.5% glutaric dialdehyde for 1.5 h at 4 °C. After being washed with PBS for three times, 100 μL 0.1% crystal violet were filled into wells for dyeing and incubated for 15 min. Following removal of the crystal violet solution, the plates were washed with PBS and dried. The crystal violet was dissolved in 200 μL 95% ethanol for 30 min. The value of OD570 nm was measured, and the blank control was TSB medium with the same operation [33]. Identification by crystal violet staining: the mean OD570 of the blank control was ODc, OD was the mean value of tested strain, and the biofilm formation ability of the strain was divided into four categories: negative (-): OD < ODc; A small amount (1 +): ODc < OD ≤ 2 ODc; Medium (2 +): 2 ODc < OD ≤ 4 ODc; A large amount (3 +): OD > 4 ODc.

2.6.2. Effect on Early Biofilms Formation

Mid-log phase *S. agalactiae* ATCC 13813 cells were diluted to 1×10^8 CFU/mL by TSB medium. A 180 μL bacterial suspension and a range of concentrations (0.25–8 × MIC) of CHAP$_k$ (20 μL) were tested and the most effective concentration was chosen for the subsequent biofilm assays. The plates were cultured for early biofilms formation for 24 h at 37 °C, then the effect of CHAP$_k$ on biofilms formation was evaluated by crystal violet staining, as described above [33]. The untreated bacteria were used as control (A). The inhibition effect of CHAP$_k$ on biofilms was determined by the following equation: Biofilms (%) = [(A-A$_{CHAPk}$)/A] × 100.

2.6.3. Effect on Mature Biofilms

S. agalactiae ATCC 13813 cells (1×10^8 CFU/mL) were cultured in TSB medium in 96-well plates at 37 °C for 24 h. Following the final concentration of 0.25–8 × MIC CHAP$_k$ were mixed into the plates and cultured for another 24 h, the plates were cultured for mature biofilms formation and dyed by crystal violet, as described above [33].

2.6.4. Effect on Bacteria in Early Biofilms

The mid-log phase *S. agalactiae* ATCC 13813 cells (1×10^8 CFU/mL) were inoculated into in 96-well plates and cultured for 24 h at 37 °C. Plates were washed twice by PBS. Subsequently, the final concentration of 0.5–64 × MIC CHAP$_k$ or vancomycin were added into plates for 2 h at 37 °C, with PBS added wells as negative control. The plates were treated with ultrasound for 5 min, and the viable bacteria were counted on the TSA plates [33].

2.6.5. Effect on Bacteria in Mature Biofilms

Bacteria in mature biofilms were obtained by the following operation: the *S. agalactiae* ATCC 13813 cells (1×10^8 CFU/mL) were incubated in 96-well plates (200 μL/well) for 24 h, and the planktonic bacteria were rinsed by PBS. Biofilms were incubated in 200 μL TSB containing 25 × MIC vancomycin for another 24 h at 37 °C, then the biofilm-encased bacteria were counted after the planktonic bacteria were gently removed by PBS. Subsequently, biofilms were treated with 8 × MIC of CHAP$_k$ and vancomycin for 24 h at 37 °C, and the same volume of PBS was treated as control (CK), and viable bacteria in biofilms were counted by colonies counting on TSA plates [33].

2.6.6. Observation of Biofilms by Confocal Laser Scanning Microscopy (CLSM)

S. agalactiae ATCC 13813 cells (1×10^8 CFU/mL) were seeded in a confocal dish, $CHAP_k$ or vancomycin were added into the dish at the final concentration of $8 \times MIC$ and incubated for 24 h, the bacteria treated without $CHAP_k$ or vancomycin were used as control. Planktonic bacteria were gently rinsed twice, and the biofilms were dyed with SYTO9 and propidium iodide (PI) (LIVE/DEAD BacLight Bacterial Viability Kit) for 15 min. After being washed with PBS, the biofilms were observed by Zeiss LSM880 confocal microscope [33].

2.7. Statistical Analysis

All data were analyzed by GraphPad Prism 6 and the results are presented as means ± standard deviation (SD). A *p*-value of <0.05 was considered statistically significant.

3. Results

3.1. Expression and Purification of $CHAP_k$

$CHAP_k$ nucleotide sequence was successfully inserted into the *Xho*I and *Nco*I sites of pET28a to construct the pET28a-$CHAP_k$ plasmid after being confirmed by DNA sequencing (Figure 1a). The results of SDS-PAGE analysis showed that the $CHAP_k$ with C-terminal $8 \times$ His-tag was purified by a one-step affinity chromatography, and the target peak band was about 19 kDa (Figure 1b). The MALDI-TOF MS analysis showed that the molecular mass of $CHAP_k$ is 19.701 kDa, which is consistent with its theoretical value of 19.683 kDa. The yield and purity of the $CHAP_k$ were 14.6 mg/L and 95%, respectively. These results indicated that $CHAP_k$ is successfully expressed in *E. coli*.
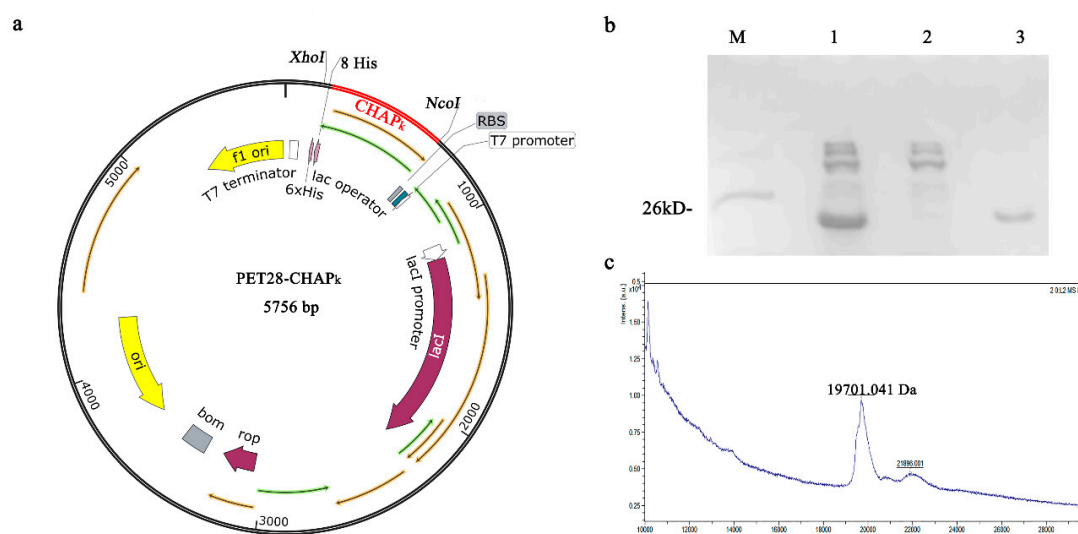
Figure 1. Expression and purification of $CHAP_k$ in *E. coli*. (**a**) The schematic diagram of the pET28a-$CHAP_k$ expression vector. (**b**) SDS-PAGE analysis of the purified $CHAP_k$. M: Protein ladder, lane 1: total protein of *E.coli* BL21-pET28a-$CHAP_k$, lane 2: the peak of penetration, lane 3: the peak of elution. (**c**) MALDI-TOF MS analysis of the purified $CHAP_k$.

3.2. Antibacterial Activity Assay

The antimicrobial activity of $CHAP_k$ was determined by MICs assay (Table 1). The MIC values of $CHAP_k$ against *S. agalactiae* were 0.05–0.11 µM, which were better than those of vancomycin (0.67 µM). $CHAP_k$ also had the potent antimicrobial activity against other streptococcus, with MICs of 0.03–0.11 µM against *S. uberis*, 0.11–0.22 µM against *S. dysgalactiae*, 0.05–0.86 µM against *S. epidermidis*, 0.11 µM against *S. suis*, which were superior to those of vancomycin (streptococcus: 0.34–2.69 µM). For the *S. aureus* strains, the MIC values of $CHAP_k$ were 0.43–3.44 µM, that of *S. hyicus* were 1.72 µM, those

of *S. epidermidis* were 0.86–1.72 μM. There was almost no antimicrobial activity against Gram-negative and *C. albicans* (>6.88 μM). These results suggested that $CHAP_k$ displays potent antimicrobial activity for staphylococcus and streptococcus, especially for streptococcus.

Table 1. The MIC values of $CHAP_k$ and vancomycin.

| Strain	MIC			
	$CHAP_k$		Vancomycin	
	μg/mL	μM	μg/mL	μM
Gram-positive bacteria				
Streptococcus agalactiae ATCC 13813	2	0.11	1	0.67
S. agalactiae CAU-FRI 1	1	0.05	1	0.67
S. agalactiae CAU-FRI 2	1	0.05	1	0.67
S. agalactiae CAU-FRI 3	1	0.05	1	0.67
S. agalactiae CAU-FRI 4	2	0.11	1	0.67
Streptococcus uberis CAU-FRI 1	2	0.11	0.5	0.34
S. uberis CAU-FRI 2	0.5	0.03	0.5	0.34
S. uberis CAU-FRI 3	1	0.05	0.5	3.34
Streptococcus dysgalactiae CAU-FRI 1	4	0.22	0.5	3.34
S. dysgalactiae CAU-FRI 2	2	0.11	1	0.67
S. dysgalactiae CAU-FRI 3	4	0.22	0.5	0.34
Streptococcus pneumoniae CVCC 3309	1	0.05	1	0.67
S. pneumoniae CVCC 2350	8	0.43	4	2.69
S. pneumoniae CGMCC 1.8747	16	0.86	4	2.69
Streptococcus suis CVCC 606	2	0.11	1	0.67
S. suis CVCC 3928	2	0.11	1	0.67
Staphylococcus aureus KY	8	0.43	4	2.69
S. aureus KR	8	0.43	4	2.69
S. aureus FJ	32	1.72	8	5.38
MRSA *S. aureus* ATCC 43300	64	3.44	8	5.38
S. aureus CVCC 546	64	3.44	8	5.38
Staphylococcus hyicus NCTC 10350	32	1.72	4	2.69
Staphylococcus epidermidis ATCC 12228	32	1.72	4	2.69
S. epidermidis ATCC 35984	16	0.86	4	2.69
Gram-negtive bacteria				
Escherichia coli ATCC 25922	>128	>6.88	>128	86.14
Salmonella enteritidis CVCC 3377	>128	>6.88	>128	86.14
Fungi				
Candida albicans CGMCC 2.2411	>128	>6.88	>128	86.14

3.3. Time-Killing Kinetic Curves in TSB and Milk

Assessment of bactericidal activity of $CHAP_k$ was performed by time-killing kinetic curves. This is an important indicator of efficacy evaluation, indicating the bactericidal rate of $CHAP_k$. The results showed that $CHAP_k$ displayed rapid bactericidal activity. With 16 × MIC $CHAP_k$ incubation, the bacteria number were reduced by 1.7 $\log_{10}$ CFU/mL in 1 min, which was four times higher than that of vancomycin (0.4 $\log_{10}$ CFU/mL). $CHAP_k$ reduced bacteria by 3.3 $\log_{10}$ CFU/mL within 25 min, whereas that of vancomycin was 0.9 $\log_{10}$ CFU/mL, which showed significant differences in bacteria kiling activity between $CHAP_k$ and vancomycin groups ($p < 0.001$) (Figure 2a,b). Considering the potential use of $CHAP_k$ as mastitis therapeutics, the antimicrobial activity of $CHAP_k$ in milk was evaluated. $CHAP_k$ also retained high activity in fresh milk, which caused 2.4 $\log_{10}$ CFU/mL reduction of S. agalactiae within 25 min, however, vancomycin showed only 0.9 $\log_{10}$ CFU/mL reduction.
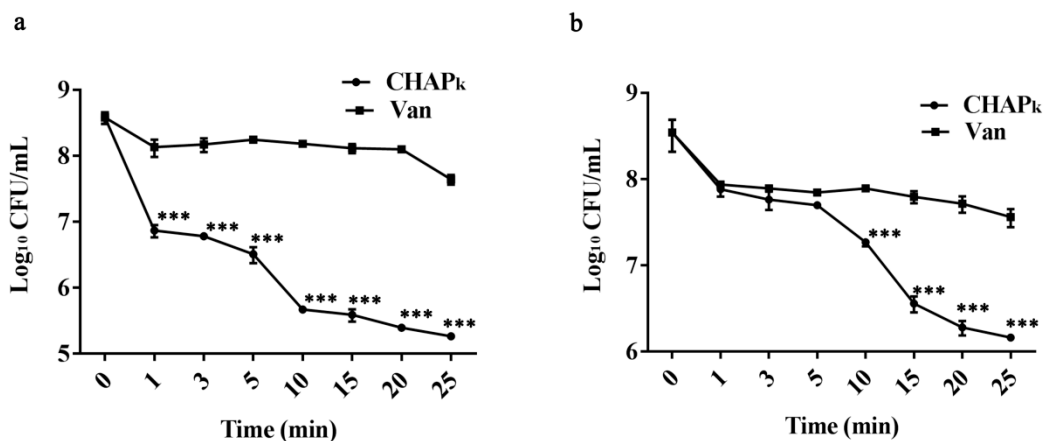
Figure 2. Time-killing kinetic curves in TSB and milk (**a**) Time-killing kinetic curves in TSB medium. (**b**) Time-killing kinetic curves in milk. All assays were performed in triplicate. The analyses were measured by one-way ANOVA, with Duncan's multiple comparisons test. A probability value of <0.05 was considered significant. (*) Indicates the significance between control and treatment groups. ***$p < 0.001$. The results are given as the mean ± SD ($n = 3$).

3.4. SEM Observation

The changes in cell morphology, integrity, and cellular structure of *S. agalactiae* ATCC 13813 were directly observed by SEM after treatment with CHAP$_k$ or vancomycin. The untreated bacteria were ovoid and clustered together, displaying morphologic integrality, and the cell surface was smooth (Figure 3). After treatment with vancomycin, wrinkles on the cell surface were observed, some cells were slightly deformed, but most of the cells remained intact. However, after treatment with CHAP$_k$, the number of bacteria was significantly reduced, cells wrinkled seriously and even ruptured, implying that CHAP$_k$ and vancomycin exhibit different bactericidal mechanisms.
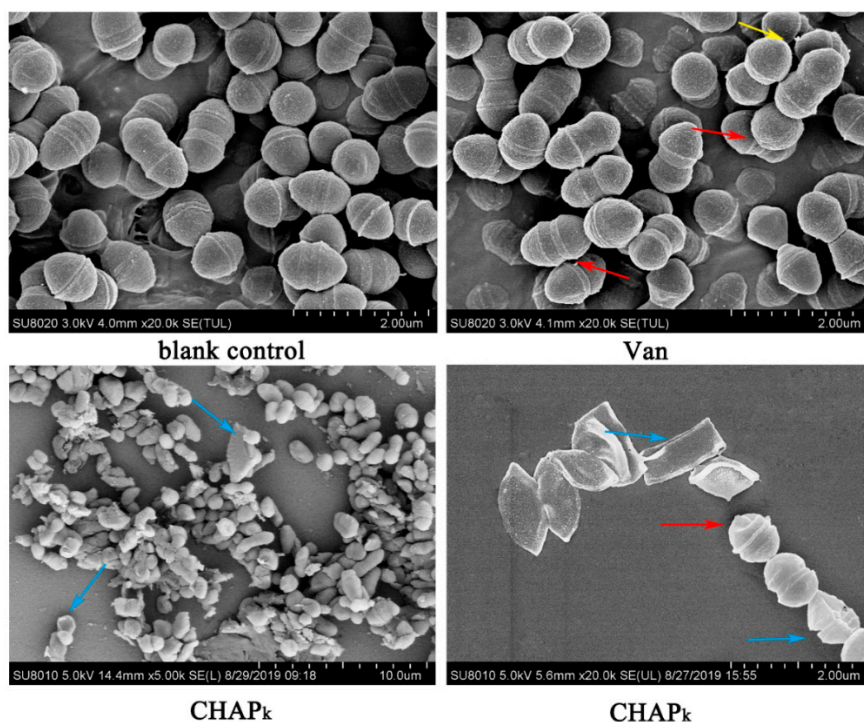
Figure 3. Scanning electron microscope observation. The magnification of the image is 20,000 (blank control), 20,000 (Van), 5000 (CHAP$_k$-left bottom), and 20,000 (CHAP$_k$-right bottom), respectively. Red arrows: Cell shrinkage; Yellow arrows: Vesicular bulge; Blue arrows: Cell rupture; Van: vancomycin.

3.5. Ability of CHAP$_k$ against Biofilms and Bacteria of S. agalactiae

3.5.1. Biofilms Formation Capacity of S. agalactiae ATCC 13813

According to the evaluation criteria of biofilms formation capacity based on crystal violet staining method, the absorbance value of *S. agalactiae* ATCC 13813 (0.361 ± 0.04) was four times higher than that of blank control (0.089 ± 0.01) at OD570 nm, which indicated that *S. agalactiae* ATCC 13813 is a strong biofilm-forming strain.

3.5.2. Effect of CHAP$_k$ on S. agalactiae ATCC 13813 Early and Mature Biofilms

Early biofilm showed obvious signs of cell-cell aggregation. To investigate the inhibitory effect of CHAP$_k$ on early biofilms, different concentrations of CHAP$_k$ were exposed to *S. agalactiae*. After treatment with 0.25–8 × MIC CHAP$_k$ or vancomycin, the biofilms were inhibited by 67.3–95% and 67.3–90.9%, respectively, whose inhibitory effect was displayed in a concentration-dependent manner (Figure 4a). These data suggested that CHAP$_k$ has a potent inhibition ability to early biofilms, which is similar to vancomycin.
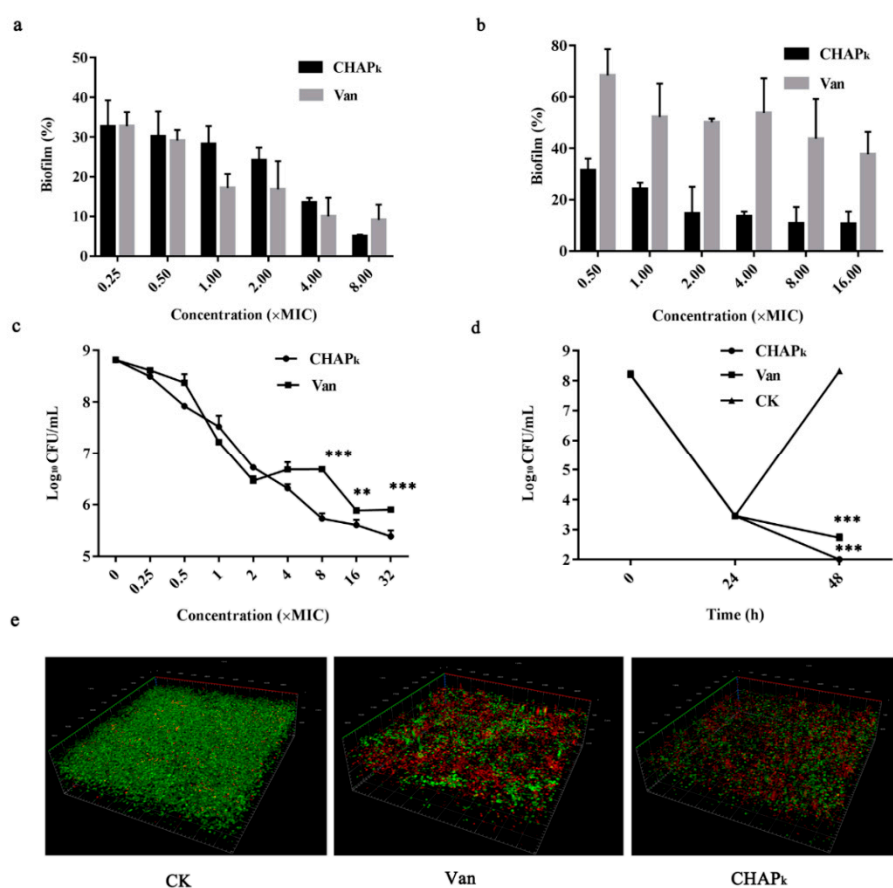
Figure 4. Effects of CHAP$_k$ on *S. agalactiae* biofilms and bacteria in biofilms. (**a**) Inhibition of biofilms formation by CHAP$_k$. (**b**) Eradication of mature biofilms by CHAP$_k$. (**c**) Bactericidal activity against the early biofilms of *S. agalactiae*. (**d**) Bactericidal activity of CHAP$_k$ against the mature biofilms of *S. agalactiae*. (**e**) Observation of *S. agalactiae* biofilms by CLSM. *S. agalactiae* was incubated with 8 × MIC CHAP$_k$ or vancomycin for 24 h; after removing planktonic bacteria, biofilms were stained with dyes and visualized by CLSM. Live cells are stained in green by SYTO9 and dead cells are stained in red by PI, CK: the untreated *S. aureus* biofilms. Van: vancomycin. All assays were performed in triplicate. The analyses were measured by one-way ANOVA, with Duncan's multiple comparisons test. A probability value of < 0.05 was considered significant. (*) Indicates the significance between control and treatment groups. ** $p < 0.01$; *** $p < 0.001$. The results are given as the mean ± SD ($n = 3$).

Peptidoglycan further bound and fully deployed to form mature biofilms. Mature biofilms are more difficult to remove than early biofilms. The eradication rate of mature biofilms was only 31% after exposed to $0.5 \times$ MIC vancomycin, which was much lower than that of early biofilms. After treatment with $16 \times$ MIC vancomycin, 62.3% mature biofilms were eradicated (Figure 4b). At the concentration of $0.5–16 \times$ MIC $CHAP_k$, the eradication rate of mature biofilms reached 68.4–89.4%, which was superior to that of vancomycin. $CHAP_k$ eradicated the mature biofilms in a concentration-dependent manner. It demonstrated that $CHAP_k$ has a stronger ability to eradicate the mature biofilms than vancomycin.

3.5.3. Effect on Bacteria in Early and Mature Biofilms

The growth of bacteria in early biofilms was investigated by plate counting. *S. agalactiae* ATCC 13813 cells in early biofilms significantly declined after exposure to $CHAP_k$ (Figure 4c). At the concentrations of $0.25–4 \times$ MIC, there were no significant differences in bacterial reduction between $CHAP_k$ and vancomycin, but at the concentrations of $8–32 \times$ MIC, the bacterial reduction caused by $CHAP_k$ was superior to that of vancomycin, and there were significant differences between $CHAP_k$ and vancomycin groups ($p < 0.01$). The results indicated that $CHAP_k$ can effectively kill bacteria in early biofilms of *S. agalactiae* in a concentration-dependent manner, which was in accordance with results of its inhibitory effect on biofilms in early stages.

To further explore the activity of $CHAP_k$ against bacteria in mature biofilms which were resistant to vancomycin, the mature biofilms of *S. agalactiae* ATCC 13813 were exposed to $25 \times$ MIC vancomycin for 24 h, the bacteria encapsulated in biofilms reduced from 10^8 CFU/mL to 10^3 CFU/mL and no colonies were regrown. The bacteria in mature biofilms were significantly killed by 99% with $4 \times$ MIC $CHAP_k$ in 24 h, which was superior to vancomycin (Figure 4d). It indicated that $CHAP_k$ has the potential activity to kill the vancomycin-resistant bacteria.

3.5.4. Observation of Biofilms by CLSM

To further confirm the inhibition and eradication effects of biofilms and internal bacteria, the *S. agalactiae* ATCC 13813 cells were treated with SYTO9 and PI, and observed by CLSM (Figure 4e), the thickness of biofilms formed by untreated *S. agalactiae* in confocal dish reached 20.72 μm. Compared with untreated group, the biofilms significantly became thinner (thickness of 11.28 μm) and dead bacteria increased in $CHAP_k$ treatment group, which was superior to vancomycin treatment group, implying the strong activity of $CHAP_k$ against *S. agalactiae* and the biofilms.

4. Discussion

S. agalactiae is a contagious pathogen which mainly causes bovine intramammary infections and spreads to the herd [34–38]. With the increase of antibiotics resistance, especially for β-lactam antibiotics [8], novel antimicrobial agents are urgently needed. More recently, the original application of phage as therapeutics to treat human and animal infections has been rekindled, lysins have attracted attention again due to their specific antimicrobial activity after being discovered for a century [12,39]. Deeper research is now involved in lysins, especially for their effect on Gram-positive bacteria. $CHAP_k$ is reported truncated phage lysin and it presents a broader antimicrobial spectrum as same as the original LysK enzyme. Compared with native enzyme LysK, the truncated single-domain lysin, $CHAP_k$, showed the same lytic activity [22]. $CHAP_k$ displays strong inhibition activity against *S. aureus*, including methicillin resistant *S. aureus* (MRSA) and vancomycin-intermediate *S. aureus* (VISA) strains [19,22–26]. $CHAP_k$ also has some antibacterial effects against streptococcus [21]. In this study, it was firstly demonstrated that $CHAP_k$ had the anti-biofilm and inhibition of bacteria in biofilms effects on *S. agalactiae.* In this study, $CHAP_k$ has potent antibacterial activity against *S. aureus*, the MIC values were 0.43–3.44 μM (Table 1), this result is consistent with the conclusions of former research in this field, which demonstrated the antimicrobial activity against *S. aureus* in vitro [19,21,22,25,40]. Meanwhile, $CHAP_k$ also displayed antimicrobial activity for *S. agalactiae* isolated from bovine mastitis. Previous studies have demonstrated that $CHAP_k$ can rapidly lyse *S. aureus*, for *Streptococcus* (*S. mutans*

DSM 6178 and *S. pneumoniae* DSM 11865), small decrease in turbidity was observed after treatment with $CHAP_k$, which means less cell death [26], but little research in this field about *S. agalactiae*. In this study, the antimicrobial activity of $CHAP_k$ has been investigated against *S. agalactiae*. $CHAP_k$ exhibited high antibacterial activity against *S. agalactiae* (MIC: 0.05–0.11 μM), and also displayed a potent effect against the other streptococcus, including *S. uberis* and *S. dysgalactiae*, which were also the pathogens causing bovine mastitis. These results suggest that $CHAP_k$ may work at a common part in the peptidoglycan cross-bridge of staphylococcal and streptococcal pathogens. $CHAP_k$ cleaves the staphylococcal cell wall on the peptide bond between D-alanine and the first glycine in the pentaglycine cross bridge [19,26], whereas the streptococcal peptidoglycan cross-bridge contains a D-alanine-L-alanine bond and no glycine residues [41]. It was reported that the Cpl-7 cell wall binding domains of the streptococcal phage lysin λSa2 were replaced by staphylococcal SH3b domains from phage lysin LysK, which resulted in increased staphylolytic activity by five times based on the maintained streptolytic activity, suggesting that the staphylococcal domains have certain broad-spectrum antibacterial properties that are not always staphylococcal-specific [42]. The activity of $CHAP_k$ against *S. agalactiae* is worth further investigation. In this study, time-killing kinetic curves showed that $CHAP_k$ had a rapid effect on bacteria both in TSB medium and in milk within 25 min, which was superior to vancomycin (Figure 2), and the result was accordance with the former research that $CHAP_k$ retained antimicrobial activity in raw bovine milk [40]. However, the effect of $CHAP_k$ was weaker in milk than that in TSB medium, this might include reduced affinity to the *S. agalactiae* cell envelope which was changed when grown in milk or $CHAP_k$ bound to milk components [43]. Because of the special mode of inhibition, lysins have no effect on commensal microflora, which is also a reason for the safety of using $CHAP_k$ [44–46]. In addition, the destructed cell wall and leaked contents indicated that $CHAP_k$ kills *S. agalactiae* by causing cells to rupture (Figure 3). The activity of $CHAP_k$ truncated cell-wall binding domain (CBD) has been suggested to be connected with the overall charge of the enzymatically active domain (EAD) alone, seemingly because EAD with positive charge can independently bind the bacterial cell wall of the CBD [44].

Biofilms provide a habitat or reservoir for bacteria, which make it difficult for antibiotics to work, leading to resistances and reinfections [47,48]. A previous study has demonstrated that $CHAP_k$ has the anti-biofilm properties for *S. aureus* [41]. In this study, $CHAP_k$ has the effect on early biofilms (24 h growth) and mature biofilms (48 h growth) in a concentration-dependent manner (Figure 4a,b) [49]. For the early biofilms, $CHAP_k$ had potent inhibited ability which is similar to vancomycin, the reason may be that in the early stage of biofilms production, low yield of biofilms is insufficient to provide protection for bacteria, antibiotics can inhibit the formation of biofilms by killing the *S. agalactiae*. However, for mature biofilms, $CHAP_k$ displayed higher eradication rate than that of vancomycin, and previous research indicated there was no changes in other streptococcus (*Streptococcus anginosus*) biofilms by vancomycin exposure [50]. The results demonstrated that $CHAP_k$ had a more potent anti-biofilm ability than vancomycin, which is consistent with the observation of biofilms by CLSM (Figure 4e). $CHAP_k$ also successfully disrupted the *S. aureus* biofilms at a concentration as low as 3.91 μg/mL, there was little or no visible biofilms detected at a concentration of 62.5 μg/mL [51]. The antibiofilm activity of chimeric phage endolysin Ply187 on biofilms of MRSA strains were measured by live/dead staining assay, which indicated effective antimicrobial activity, whereas gentamycin had a poor effect on biofilms viability [48], which indicated that the lysins have better anti-biofilm activity than antibiotics. The bacteria in biofilms is a major reason that causes reinfections. $CHAP_k$ can effectively reduce the number of *S. agalactiae* ATCC 13813 in biofilms, which showed a significant difference with vancomycin. Earlier studies have shown that vancomycin that inhibit cell wall synthesis reduce bacterial adherence, thus reducing bacteria and biofilm formation [52]. Recent research showed that the resistance of *Streptococcus anginosus* to vancomycin in a multispecies biofilm is due to increased thickness of the cell wall [50]. However, the elimination of biofilms by lysin is in connection with lysin inhibiting poly-intercellular adhesion (PIA) binding to peptidoglycan by rapidly degrading the cell wall and significantly decreasing the eDNA content. This may be the reason why $CHAP_k$ is more

potent in killing *S. agalactiae* and inhibiting biofilms formation than vancomycin [53]. The bacteria in biofilms show reduced susceptibility to antibiotics among several important pathogens [54], in contrast, lysins have high efficiency activity against bacteria in biofilms. CHAP$_k$ killed the bacteria in biofilms, which were resistant to $25 \times$ MIC vancomycin. Survival of bacteria in *S. aureus* biofilms were formed by treatment with 2 mg/mL of rifampicin or 3 mg/mL of ciprofloxacin for 4 h, which could be eliminated with 0.5 mM endolysin LysH5 [55]. After treatment with $1 \times$ MIC of P128, the survival of *S. epidermidis* in $50 \times$ MIC daptomycin or $100 \times$ MIC vancomycin could be drastically reduced [56].

In summary, recombinant CHAP$_k$ was successfully expressed in *E. coli* via pET28a, CHAP$_k$ exhibited potent antimicrobial activity against Gram-positive, especially for *S. agalactiae* which were isolated from bovine mastitis. CHAP$_k$ could rapidly lyse *S. agalactiae* cells in a short time, and it also significantly inhibited its biofilms formation at an early stage and eliminated its mature biofilms. For the bacteria in biofilms, CHAP$_k$ showed efficient antimicrobial ability, which was superior to vancomycin. The potent antimicrobial activity of CHAP$_k$ against the *S. agalactiae* isolated from bovine mastitis is the basis for its clinical application. It is suggested that CHAP$_k$ may be a candidate for novel antimicrobial agents against streptococcal and even staphylococcal infections in mastitis treatment.

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