

# The potential of the endolysin Lysdb from *Lactobacillus delbrueckii* phage phiLdb for combating *Staphylococcus aureus* during cheese manufacture from raw milk

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**Abstract** Phage endolysins have received increased attention in recent times as potential antibacterial agents and the biopreservatives in food production processes. *Staphylococcus aureus* is one of the most common pathogens in bacterial food poisoning outbreaks. In this study, the endolysin Lysdb, one of the two-component cell lysis cassette of *Lactobacillus delbrueckii* phage phiLdb, was shown to possess a muramidase domain and catalytic sites with homology to *Chalaropsis*-type lysozymes. Peptidoglycan hydrolytic bond specificity determination revealed that Lysdb was able to cleave the 6-*O*-acetylated peptidoglycans present in the cell walls of *S. aureus*. Turbidity reduction assays demonstrated that Lysdb could effectively lyse the *S. aureus* live cells under acidic and mesothermal conditions. To further evaluate the ability of Lysdb as a potential antibacterial agent against *S. aureus* in cheese manufacture, *Lactobacillus casei* BL23 was engineered to constitutively deliver active Lysdb to challenge *S. aureus* in lab-scale cheese making from raw milk. Compared with the raw milk, the viable counts of *S. aureus* were reduced by 10<sup>5</sup>-fold in the cheese inoculated with the engineered *L. casei* strain during the fermentation process, and the pathogenic bacterial numbers remained at a low level (10<sup>4</sup> CFU/g) after 6 weeks of ripening at 10 °C. Taken together, all results indicated that the Lysdb has the function as an effective tool for combating *S. aureus* during cheese manufacture from raw milk.

**Keywords** Endolysin · *Lactobacillus casei* · *Staphylococcus aureus* · Antibacterial

## Introduction

Phages are the most abundant living entities on Earth and kill 50 % of the bacteria produced every day (Oliveira et al. 2013). Double-stranded DNA phages utilize a two-component cell lysis cassette (endolysin and holin) to collapse the peptidoglycan layer of bacterial cell walls, enabling progeny release and new host infection (Young 2013). Holins are small membrane-spanning proteins that accumulate as oligomers in the cytoplasmic membrane (White et al. 2011). Endolysins are highly evolved enzymes that target bonds within the peptidoglycan (Catalão et al. 2012). There are five major functional types of phage endolysins: muramidase, amidase, peptidase, lytic transglycosylase, and endopeptidase. Muramidases hydrolyze the  $\beta$ -1,4 glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine of the bacterial peptidoglycan (Endersen et al. 2014). Several muramidases belong to a group termed the *Chalaropsis*-type (*Ch*-type) lysozymes, including those from *Chalaropsis*, *Streptomyces coelicolor*, phages of *Streptococcus pneumoniae* (Cp phages), *Lactobacillus* spp. phage MV1, and  $\varphi$ adh, which display both  $\beta$ -1,4-*N*-acetylmuramidase and  $\beta$ -1,4-*N*,6-*O*-diacetylmuramidase activity (Pérez-Dorado et al. 2007; Rau et al. 2000). The latter enables endolysin to degrade the cell wall of *Staphylococcus aureus* (Rau et al. 2000). Some endolysins only act upon the cell walls of several bacterial phylotypes, while some show broad-spectrum antimicrobial activity (Yoong et al. 2004). These properties make endolysins be effective candidates as antibacterial agents that could potentially replace antibiotics to target specific pathogens (Callewaert et al. 2011; Rodríguez-Rubio et al. 2012; Park et al. 2012; Zhang et al. 2012).

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*S. aureus* is the most prevalent food-borne pathogen of traditionally produced cheeses (Fleuret et al. 2014). During cheese production processes, raw milk from mastitic cows, biofilm in the processing plant environment, and healthy human carriers are the probable sources of *S. aureus* contamination (Walcher et al. 2014). Milk is a good substrate for *S. aureus* growth, and when *S. aureus* counts reach approximately  $10^6$  CFU/g, this species begin to produce enterotoxin (Gücükoğlu et al. 2012). Therefore, dairy products are a known source of intoxication (De Buyser et al. 2001; Jorgensen et al. 2005). To prevent the growth of *S. aureus*, several strategies have been employed to eliminate vegetative pathogenic bacteria, including thermal processing and chemical preservatives. While pathogen-free food with minimal processing, fewer chemical preservatives, and high nutritional value becomes an ever-growing demand around the world, which promotes the development of new natural preservatives (Castellano et al. 2015; Tilkens et al. 2015). Accordingly, phage endolysins have drawn increasing interests as novel natural preservatives to treat food-borne *S. aureus* (Obeso et al. 2008).

Lactic acid bacteria (LAB), widely used in food fermentation, are generally recognized as safe (GRAS) status and chosen to heterologously produce endolysins (Hugenholtz 2008; Morello et al. 2008). *Lactococcus lactis* has been used to synthesize endolysins Ply511 (*N*-acetylmuramoyl-l-alanine amidase) and Ply118 (l-alanoyl-d-glutamate peptidase) from *Listeria monocytogenes* bacteriophages in an effort to develop dairy starter strains with food biopreservation properties (Gaeng et al. 2000). The endolysin LysK from staphylococcal bacteriophage K was expressed in *L. lactis* as an alternative to *Escherichia coli* (O'Flaherty et al. 2005). Also, secretion of active *Clostridium perfringens* endolysin CP25L by *Lactobacillus johnsonii* F19785 has been achieved for biocontrol of *C. perfringens* in the simulative gastrointestinal tract (Gervasi et al. 2014a). *Lactobacillus casei* is a major non-starter lactic acid strain in cheese making, and also applied as an ideal host for the production of bioactive enzymes in the manufacture of various dairy products (Rochat et al. 2006; Xu and Kong 2013). In this study, an endolysin Lysdb, originating from *Lactobacillus delbrueckii* phage phiLdb was identified as a *Ch*-type lysozyme with lytic activity against *S. aureus*, and this endolysin was constitutively secreted by *L. casei* BL23 for the biocontrol of *S. aureus* during cheese manufacture from raw milk.

## Materials and methods

### Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *L. delbrueckii* subsp. *bulgaricus* ATCC11842 was kindly provided by Prof. M. van de Guchte and used to propagate the virulent phage phiLdb (van de Guchte et al. 2006;

Wang et al. 2010). *Lactobacillus* was grown in de Man, Rogosa and Sharpe (MRS) broth at 37 °C statically. *L. lactis* and *Enterococcus faecalis* were grown in M17 broth (Oxoid, UK) supplemented with 0.5 % (wt/vol) glucose (GM17) at 30 °C statically. *Streptococcus thermophilus* was grown in M17 broth supplemented with 1 % (wt/vol) lactose at 42 °C statically. *E. coli*, *S. aureus*, *Bacillus cereus* and *Salmonella enterica* subsp. *enterica* serovar *Braenderup* were grown in LB broth at 37 °C aerobically. *L. monocytogenes* strains were grown in brain heart infusion (BHI) broth at 37 °C aerobically. When appropriate, the following antibiotics were added to the broth or the agar medium: ampicillin (100 µg/mL for *E. coli*) and chloramphenicol (10 µg/mL for *E. coli* or 2.5 µg/mL for *Lactobacillus*).

### Identification and cloning of the putative endolysin gene *lysdb* from phage phiLdb

The endolysin sequence is available in the phage phiLdb complete nucleotide sequence under GenBank accession number KF188410. The conserved domain search was conducted using the NCBI Conserved Domain Database (<http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi>). Amino acid alignments were performed using ClustalW software. Tertiary structure analysis was conducted using the Swiss Model (<http://swissmodel.expasy.org>) online server.

Molecular cloning techniques were performed using standard methods (Sambrook and Russell 2001). *Taq* DNA polymerase, restriction enzymes, T4 DNA polymerase, and T4 DNA ligase were used according to the instructions of the manufacture (TaKaRa, Japan).

The putative endolysin gene *lysdb* was PCR amplified from the genomic DNA of phiLdb (Wang et al. 2010) with primers PLysdb-NdeIF (5'-TGACATATGACTAAGTTT GATTTAATGGCTG-3'; the underlined bases indicate an *NdeI* site) and PLysdb-XhoIR (5'-ATCCTCGAGACT GAATTTACCCCAATAA-3'; the underlined bases indicate an *XhoI* site). The PCR product was restricted with *NdeI*/*XhoI* and cloned into the corresponding sites of the pET-22b(+) vector. The resultant plasmid pELysdb was transformed into chemically competent *E. coli* BL21 cells, generating the recombinant strain *E. coli*/pELysdb.

### Analysis of the lytic activity of Lysdb

To prepare the purified Lysdb protein, *E. coli*/pELysdb was grown at 37 °C aerobically to an OD<sub>600</sub> of 0.8, and then 0.1 mM isopropyl-β-D-thiogalactopyranoside was added to induce the expression of the His-tagged protein. The His-tagged Lysdb protein was purified by Ni-NTA affinity chromatography. The purified protein was dialyzed against 20 mM sodium phosphate (pH 6.0) and quantified using the Bradford protein assay. The lytic activity of Lysdb against the indicator strains was tested by zymogram as described previously (Wang et al. 2008).

**Table 1** Bacterial strains and plasmids used in this study

| Strain, phage, or plasmid  | Relevant characteristic(s) and description  | Source                      |
|--|---|-----------------------------|
| <b>Strains</b>   |   |                             |
| <i>Escherichia coli</i> DH5 $\alpha$   | $F^-$ <i>supE44</i> $\Delta$ <i>lacU169</i> $\Phi$ 80 <i>lacZ</i> $\Delta$ <i>M15</i> <i>hsdR17</i><br><i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>relA1</i>                | Novagen                     |
| <i>Escherichia coli</i> BL21 (DE3)   | $F^-$ <i>ompT</i> <i>hsdS<sub>B</sub></i> ( $r_B^-$ $m_B^-$ ) <i>gal</i> <i>dcm</i> (DE3)   | Novagen                     |
| <i>Lactobacillus delbrueckii</i> subsp. <i>bulgaricus</i> ATCC11842                        | Host bacteria of virulent phage phiLdb  | (van de Guchte et al. 2006) |
| <i>Lactobacillus casei</i> BL23  | Commonly used lactobacilli for NICE system  | (Hazebrouck et al. 2007)    |
| <i>Lactobacillus brevis</i> ATCC367  |   | Our lab                     |
| <i>Lactobacillus plantarum</i> WCFS1   |   | Our lab                     |
| <i>Lactococcus lactis</i> subsp. <i>lactis</i><br>bv. <i>diacetylactis</i> CCTCCAB20102111 | Wild-type strain  | Our lab                     |
| <i>Streptococcus thermophilus</i> CGMCC 7.179  | Wild-type strain  | Our lab                     |
| <i>Enterococcus faecalis</i> ATCC14506   |   | Our lab                     |
| <i>Staphylococcus aureus</i> ATCC33591   | Resistant methicillin   | Our lab                     |
| <i>Staphylococcus aureus</i> ATCC27217   | Penicillin sensitive, resistant to 5 $\mu$ g but<br>sensitive to 10 $\mu$ g of tetracycline   | Our lab                     |
| <i>Listeria monocytogenes</i> ATCC19114  | Antigenic propertie, Serotype 4a  | Our lab                     |
| <i>Bacillus cereus</i> ATCC33019   | Food-borne pathogen   | Our alb                     |
| <i>Salmonella enterica</i> subsp. <i>enterica</i><br>serovar <i>Braenderup</i> H9812       | Food-borne pathogen   | Our alb                     |
| <b>Plasmids</b>  |   |                             |
| pET-22b(+)   | Expression vector; T7 promoter; Amp <sup>r</sup>  | Novagen                     |
| pSec:Nuc   | Usp45 signal peptide fused to the staphylococcal<br>nuclease gene ( <i>nuc</i> ) mature moiety under the<br>control of the nisin-inducible promoter P <sub>nisA</sub> ; Cm <sup>r</sup> | (Enouf et al. 2001)         |
| pELysdb  | <i>lysdb</i> cloned in pET22b; Amp <sup>r</sup>   | This study                  |
| pSLysdb  | <i>lysdb</i> cloned in pSec:Nuc; Cm <sup>r</sup>  | This study                  |
| pBLysdb  | Promoter P <sub>nisA</sub> was replaced by promoter B6<br>in pSLysdb; Cm <sup>r</sup>   | This study                  |
| pB6  | Derivate of plasmid pBLysdb with <i>lysdb</i> gene<br>deletion; Cm <sup>r</sup>   | This study                  |

To detect the lytic activity of Lysdb against *S. aureus* strains ATCC33591 and ATCC27217, exponential cells were collected, washed and resuspended in 20 mM sodium phosphate (pH 6.0) as the live cell substrates. The activity of Lysdb was assessed by turbidity reduction using 10  $\mu$ g/mL Lysdb in 20 mM sodium phosphate buffer for 100 min. The boiled Lysdb protein in the same buffer was used as the control. The relative lytic activity was calculated using the following equation:  $100 \times (\text{OD}_{600}$  of control sample –  $\text{OD}_{600}$  of reaction sample)/ $\text{OD}_{600}$  of control sample. The lytic activity was determined at temperatures ranging from 4 to 45 °C and over a pH range of 3.0 to 5.0 in 20 mM sodium citrate buffer and pH 6.0 to 8.0 in 20 mM sodium phosphate buffer.

#### Determination of the peptidoglycan hydrolytic bond specificity of Lysdb

Peptidoglycan extraction from an exponentially growing culture of *S. aureus* ATCC33591 and determination of hydrolytic bond specificity were performed according to the protocol

described by Atrih et al. (1999). The peptidoglycan (3 mg) was incubated with 500  $\mu$ g purified Lysdb protein in a final volume of 500  $\mu$ L (20 mM sodium citrate buffer, pH 4.0) overnight at 37 °C. Peptidoglycan cleavage site determination was carried out by reverse-phase high-performance liquid chromatography (RP-HPLC) and mass spectrometry (MS). Samples were applied to an XBridge BEH300 C18 reverse-phase column (150  $\times$  4.6 mm; Waters, USA) with a flow rate of 1.0 mL/min. The column was equilibrated at 45 °C with solvent A. Separation of the reduced muopeptides was effected with a gradient of 0 to 100 % solvent B over 100 min. Solvent A was 40 mM sodium phosphate (pH 4.5); solvent B was 40 mM sodium phosphate (pH 4.0) containing 20 % methanol. The column effluent was monitored by the  $A_{202}$ .

#### Constitutive secretion of Lysdb in *L. casei* BL23

A constitutive secretory vector was constructed to deliver Lysdb in *L. casei* BL23 according to following method. The *lysdb* gene was PCR amplified from the genomic DNA of



**Fig. 1** Sequence alignment of the N-terminal domain of Lysdb with *Ch-type* lysozymes from the fungus *Chalaropsis*, *Streptomyces erythraeus*, *Streptomyces cellosyl*, *Streptomyces erythraeu*, and *Xylella fastidiosa* (Rau et al. 2000). Sequence conservation is represented by *light gray* (conserved residues in three sequences), *dark gray* (conserved residues in four sequences), or *black* (equivalent residues in all sequences). Conserved catalytic active residues are indicated by *frames*

|                                |          |           |          |          |         |         |          |          |     |
|--------------------------------|----------|-----------|----------|----------|---------|---------|----------|----------|-----|
| Lysdb                          | MTKFDLMA | IVSSYQLD  | TEFFQAL  | KDKGVK   | AVKITEG | SNPGSA  | VNPKAK   | NQIKNARA | 60  |
| <i>Chalaropsis</i>             | ...TVQGF | IVSSYQPSV | N.FAGAYS | SAGARFV  | IKKATEG | ...TSW  | TNPSFSSQ | YNGATT   | 51  |
| <i>Streptomyces cellosyl</i>   | DTSGVQ   | GLVSHW    | QGSIN    | ..WSSVKS | AGMSFAY | IKKATEG | ...TNV   | KDRFSA   | 54  |
| <i>Streptomyces erythraeus</i> | ..ATVAG  | GLVSGH    | QRNVD    | ..WQY    | WVQGR   | FAYVKA  | TEG      | ...TG    | 52  |
| <i>Xylella fastidiosa</i>      | ..MFNKG  | GLVSGR    | NGEID    | ..FTK    | VEAEI   | GYVFMK  | ATEG     | ...AT    | 52  |
| Lysdb                          | AG...LKV | HAYHAK    | FNGVAD   | AAEA     | ADWFV   | VKN     | AKKLG    | .IGED    | 115 |
| <i>Chalaropsis</i>             | ATGNY    | FIRGGY    | FAHPG    | .ETTGA   | AQADY   | IAHGGG  | WSDG     | ITLPG    | 110 |
| <i>Streptomyces cellosyl</i>   | AG...IIR | GAYH      | FARRN    | .ASSG    | TAQADY  | FASNGG  | WSDR     | NRTLPG   | 110 |
| <i>Streptomyces erythraeus</i> | IG...MIR | GAYH      | FALPD    | .RSSG    | AAQAN   | YFVD    | NGG      | WSDG     | 108 |
| <i>Xylella fastidiosa</i>      | AG...MTL | GAYH      | YFRAL    | .SSTPE   | AQKDN   | VNVLT   | QNGF     | NSSCE    | 108 |
| Lysdb                          | ..TADIN  | AFIQR     | VKNAG    | YPKT     | ...DIV  | SMRS    | WFEDG    | HIDR     | 168 |
| <i>Chalaropsis</i>             | LSAAS    | MVAW      | IKAF     | SDRY     | HAVT    | GRYP    | MLY      | TNPS     | 170 |
| <i>Streptomyces cellosyl</i>   | LSTTQ    | MRTW      | INDF     | HARY     | KART    | TRDV    | VLY      | TAS      | 170 |
| <i>Streptomyces erythraeus</i> | KTPAQ    | MTAW      | IKDF     | SDTY     | HART    | GRWP    | VIVT     | ..S      | 164 |
| <i>Xylella fastidiosa</i>      | VMADN    | LKLL      | LLLL     | GEEC     | IFGD    | .RKPL   | IY       | CSPN     | 167 |
| Lysdb                          | ....GLD  | ...DVG    | TWCF     | TSEY     | PVSG    | IKVD    | ....MSY  | DFK      | 200 |
| <i>Chalaropsis</i>             | GTIPG    | GGWP      | ...YQT   | IWC      | NSDAY   | YGGS    | ....NF   | INGS     | 211 |
| <i>Streptomyces cellosyl</i>   | ..TVP    | SGFP      | ...TWT   | FQ       | SATG    | RVGG    | VSDV     | DRNK     | 211 |
| <i>Streptomyces erythraeus</i> | GKLP     | YNWG      | ...FHT   | IWC      | TSSP    | IDQNS   | ....FNG  | GYDR     | 202 |
| <i>Xylella fastidiosa</i>      | ..RIP    | QTSK      | ACKS     | WSVW     | QYSS    | KGSIP   | ING      | ....D    | 205 |

conditions of *L. delbrueckii* which was grown at the acidic and mesothermal conditions.

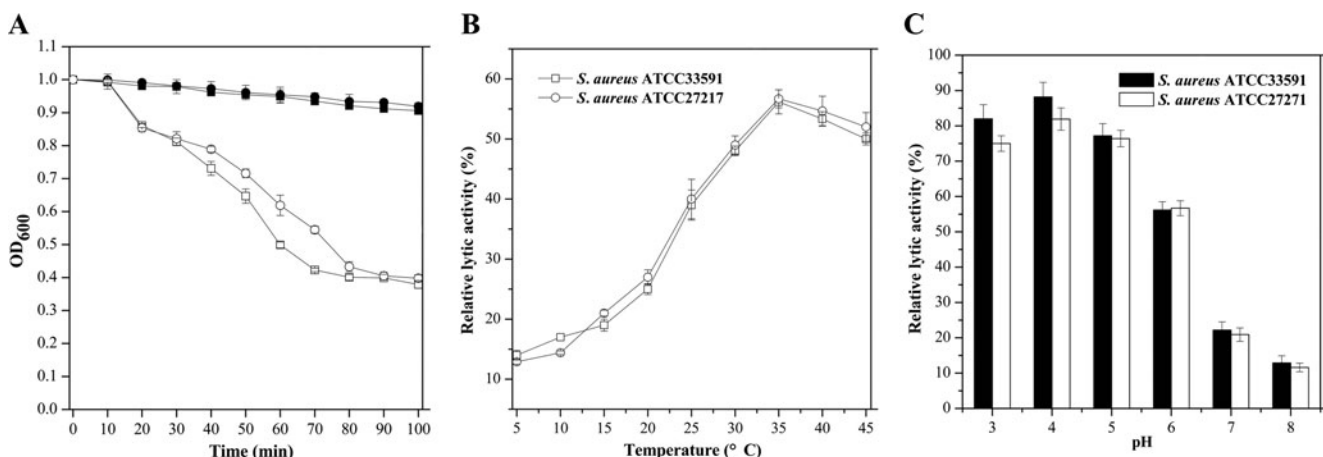
**Peptidoglycan hydrolytic bond specificity of Lysdb**

To further confirm the lytic activity of Lysdb against *S. aureus*, the peptidoglycan of the cell walls was extracted from *S. aureus* ATCC33591 cells and digested with purified Lysdb under optimal conditions. Muropeptides fragments were separated by RP-HPLC, and the molecular masses of the muropeptides were determined by LC-MS, generating ions with an *m/z* of 1008.7 and 2075.4. As shown in Fig. 3, the primary fragments of peaks 1 and 2 correspond to the disaccharide hexapeptide GlcNAc-MurNAc-(L-Ala-D-iGln-L-

Lys-(Gly)<sub>2</sub>-D-Ala) and disaccharide tetrapeptide disaccharide nonapeptide GlcNAc-MurNAc-(L-Ala-D-iGln-L-Lys-D-Ala)-(L-Ala-D-iGln-L-Lys-(Gly)<sub>5</sub>-D-Ala)-MurNAc-GlcNAc. This result demonstrated that Lysdb exhibits β-1,4-*N*,6-*O*-diacetylmuramidase activity, capable of cleaving the 6-*O*-acetylated peptidoglycans present in the cell walls of *S. aureus*.

**Constitutive delivery of active Lysdb in *L. casei* BL23**

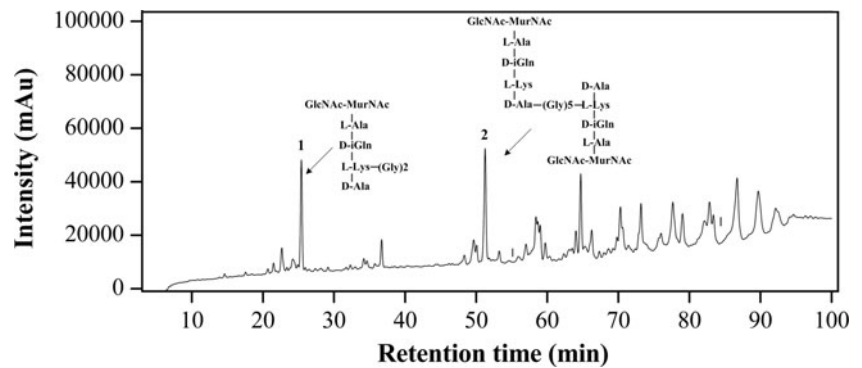
To evaluate the antibacterial activity of Lysdb during cheese making from raw milk, *L. casei* BL23, a non-starter culture of cheese was adopted to heterologously deliver Lysdb with signal peptide Usp45. A scheme illustrating the construction of



**Fig. 2** Characterization of the lytic activity of Lysdb against two *S. aureus* strains. **a** Lytic activity of Lysdb against two *S. aureus* strains at 35 °C over 100 min. *Filled squares*, *S. aureus* ATCC33591 plus 10 μg/mL boiled Lysdb protein; *empty squares*, *S. aureus* ATCC33591 plus 10 μg/mL Lysdb protein; *filled circles*, *S. aureus* ATCC27217 plus

10 μg/mL boiled Lysdb protein; *empty circles*, *S. aureus* ATCC27217 plus 10 μg/mL Lysdb protein. **b, c** Influence of temperature and pH on the lytic activity of Lysdb against two *S. aureus* strains. Results are the averages of triplicate experiments

**Fig. 3** RP-HPLC analysis of the soluble muropeptides released from *S. aureus* peptidoglycan after degradation with Lysdb

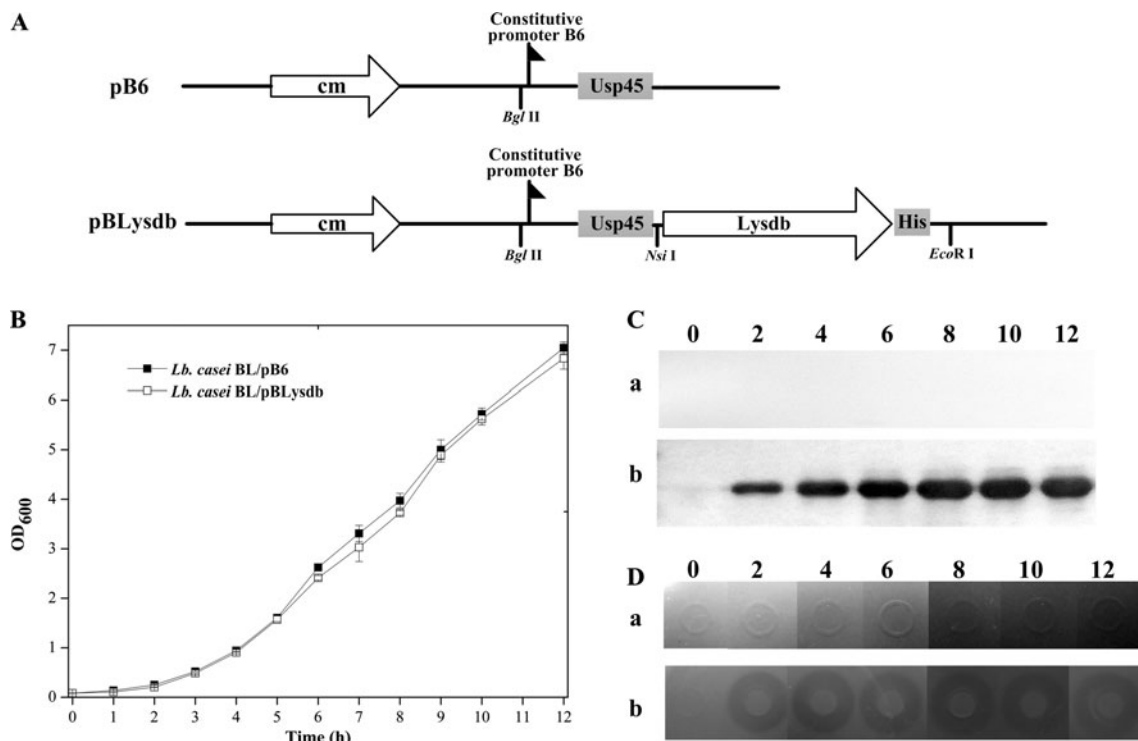


engineered *L. casei* BL/pBLysdb was shown in Fig. 4a. As a result, no obvious difference in growth was observed between the engineered strain *L. casei* BL/pBLysdb and the control strain *L. casei* BL/pB6 (Fig. 4b), indicating that the expression of Lysdb did not result in the death of host cells and the level of secretory Lysdb increased gradually during the culture time (Fig. 4c). Moreover, the supernatant of *L. casei* BL/pBLysdb produced obvious clearing zones against the autoclaved *S. aureus* cells, while no clearing zone was observed in the control group (*L. casei* BL/pB6) (Fig. 4d), indicating that

active Lysdb was successfully delivered into the supernatant by *L. casei* BL/pBLysdb.

### Challenging *S. aureus* during cheese manufacture from raw milk

Cheese is the most complex among the fermented dairy products, and *S. aureus* is a common contaminant of cheese. To evaluate the potential of the delivered Lysdb as an antibacterial agent in cheese making, a lab-scale model cheese was



**Fig. 4** Constitutive delivery of active Lysdb by *L. casei* BL/pBLysdb. **a** A scheme showing the construction of vector pBLysdb for the secretion of Lysdb in *L. casei* BL/pBLysdb, as well as the control plasmid pB6. The black line represents the pSec:Nuc backbone. To construct the plasmid pBLysdb, the nisin-inducible promoter PnisA and the *nuc* gene of the pSec:Nuc were replaced by the constitutive promoter B6 and the *lysdb* gene. To construct the plasmid pB6, the *lysdb* gene of pBLysdb was

deleted. **b** Growth curves of the control strain *L. casei* BL/pB6 and the engineered strain *L. casei*/pBLysdb in MRS broth. **c** Analysis of Lysdb in the culture supernatant of *L. casei* BL/pB6 (**a**) and *L. casei* BL/pBLysdb (**b**) by Western blot analysis. **d** Lytic activity of the culture supernatant of *L. casei* BL/pB6 (**a**) and *L. casei* BL/pBLysdb (**b**) against autoclaved *S. aureus* ATCC33591. In (**c**) and (**d**), the numbers represent the samples taken at the time points: 0, 2, 4, 6, 8, 10, and 12 h

built up using *L. lactis* CCTCCAB20102111 and *L. casei* BL/pBLysdb as the starters to challenge  $4.0 \times 10^4$  CFU/mL *S. aureus* ATCC33591 in the raw milk. As shown in Fig. 5a, after 360 min fermentation, the pH values of the experimental cheese were similar to those of the control cheese, suggesting that the model cheese worked effectively. The viable counts of *S. aureus* ATCC33591 in the raw milk drastically increased and reached  $1.7 \times 10^9$  CFU/mL after 360 min fermentation (Fig. 5b). For the control cheese group, high *S. aureus* counts appeared at 270 min with  $2.5 \times 10^7$  CFU/mL and remained constant until the end of fermentation. For the experimental cheese, the viable *S. aureus* counts increased gradually to  $1.6 \times 10^6$  CFU/mL in the first 210 min, while reduced to  $1.0 \times 10^4$  CFU/mL at the time point of 360 min, indicating that the delivered Lysdb effectively inhibited the growth of *S. aureus*. During cheese ripening at 10 °C, the viable counts of strain ATCC33591 in the raw milk and control cheese remained at a high level of  $2.0 \times 10^9$  and  $3.9 \times 10^7$  CFU/g, respectively, while the counts in the experimental cheese dropped continuously over time, reaching  $2.7 \times 10^3$  CFU/g after 6 weeks (Fig. 5c). These results indicated that the delivery of endolysin Lysdb by the engineered *L. casei* strain combated *S. aureus* efficiently during cheese manufacture from raw milk.

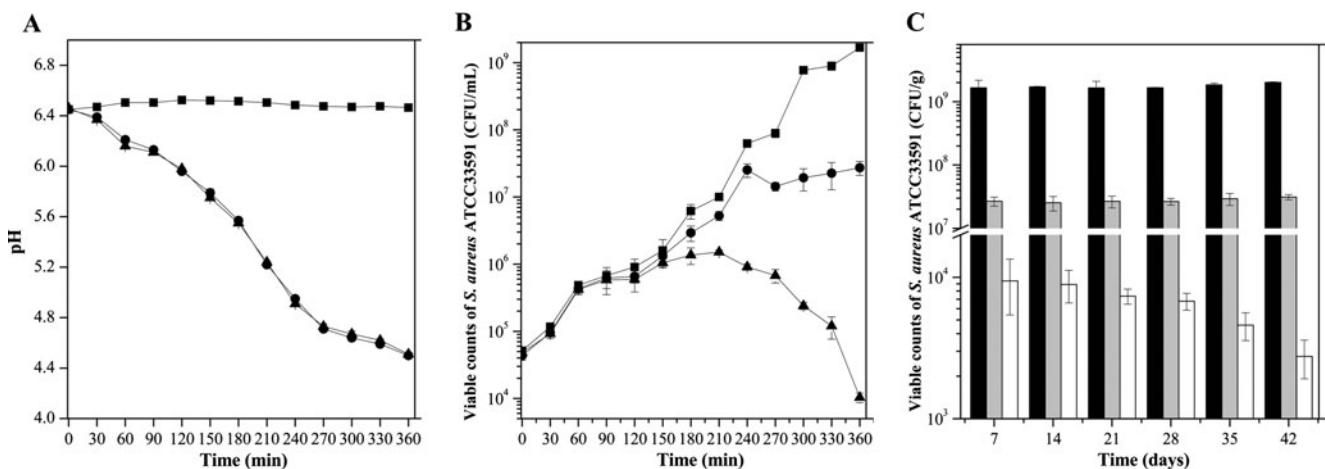
### Lytic spectrum of endolysin Lysdb

To further investigate the lytic spectrum of Lysdb as an antibacterial agent, several lactic acid bacteria commonly used in cheese making and 3-food spoilage bacteria were tested to determine their sensitivity to Lysdb. As shown in Fig. 6, Lysdb exhibited lytic activity against *L. delbrueckii*

ATCC11842, *Lactobacillus plantarum* WCFS1, *Lactobacillus brevis* ATCC367, and *E. faecalis* ATCC14506, whereas no lytic activity was observed against *L. casei* BL23, *L. lactis* subsp. *lactis* bv. diacetylactis CCTCCAB20102111, and *S. thermophilus* CGMCC 7.179. Moreover, besides two *S. aureus* strains, food spoilage organisms *L. monocytogenes* ATCC19114 and *Salmonella Braenderup* H9812 were also sensitive to Lysdb, indicating the potential of Lysdb as a biopreservative in food processes. *L. casei* and *L. lactis* are important starters and adjunct strains in cheese manufacture, and their resistance to the lytic action of Lysdb facilitates the application of this endolysin as an effective tool for combating *S. aureus* during cheese manufacture. It is known that efficient lysis requires that the C-terminus binds to its cell wall substrate (Fischetti 2008). In the case of Lysdb, its C-terminus may not be able to combine with the cell wall of *L. casei* BL23 and *L. lactis* CCTCCAB20102111, leading to their resistance to the lytic action of Lysdb.

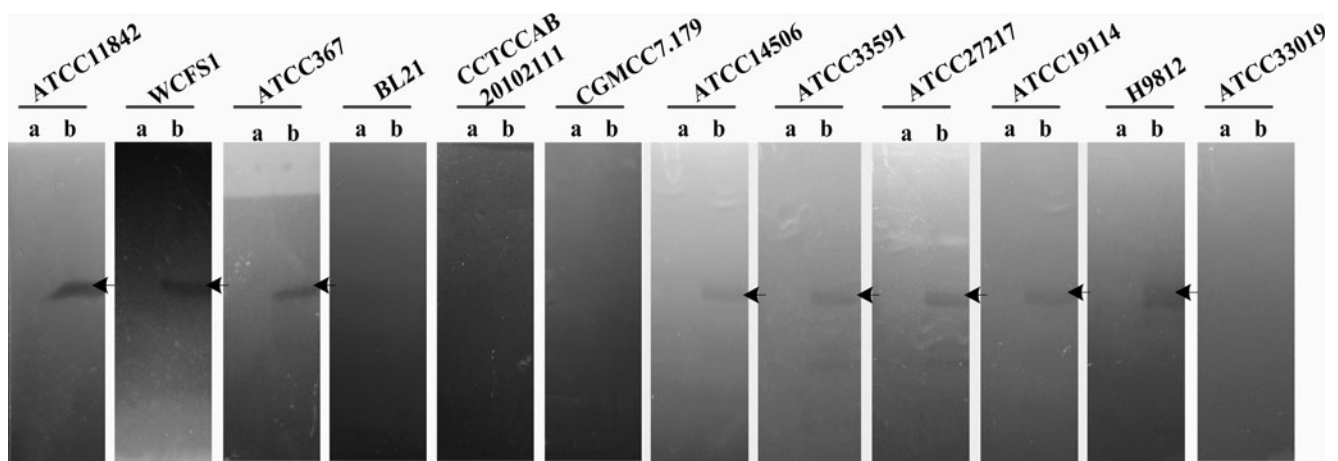
### Discussion

Despite advances in modern technologies, the food industry is continuously challenged with the threat of pathogenic and spoilage contamination. Because of the overuse of antibiotics, together with the addition of chemical preservatives, the development of new methods for controlling microbial contamination in the food industry is urgently needed (Endersen et al. 2014). Phage endolysins are considered feasible antibacterial agents for combating food spoilage (Callewaert et al. 2011). Sequence analysis showed that endolysin Lysdb contains a



**Fig. 5** Combat of *S. aureus* ATCC33591 using endolysin Lysdb delivered by *L. casei* BL/pBLysdb in raw milk model cheese. **a** pH profile of the model cheese. **b** Viability of *S. aureus* ATCC33591 during cheese fermentation. Filled squares, the raw milk; filled circles, the control cheese (the raw milk inoculated with *L. lactis* CCTCCAB20102111 and *L. casei* BL/pB6); filled triangles, the

experimental cheese (the raw milk inoculated with *L. lactis* CCTCCAB20102111 and *L. casei* BL/pBLysdb). **c** Viability of *S. aureus* ATCC33591 during cheese ripening. Black bar, the raw milk; gray bar, the control cheese; white bar, the experimental cheese. Results are the averages of triplicate experiments



**Fig. 6** Zymogram analysis of the lytic activity of Lysdb against seven LAB strains and five food-borne pathogens. **a** Boiled Lysdb protein in 20 mM sodium phosphate buffer used as a control; **b** purified Lysdb protein in 20 mM sodium phosphate buffer

catalytic domain similar to that of the *Ch*-type lysozyme at the N-terminus, which is able to cleave the 6-*O*-acetylated peptidoglycans present in the cell wall of *S. aureus*. Our experimental data also approved the lytic activity of endolysin Lysdb against *S. aureus*, demonstrating the degradation of 6-*O*-acetylated peptidoglycans in *S. aureus* cell walls by hydrolytic bond specificity assays (Fig. 3). These results confirmed the Lysdb was a member of *Ch*-type lysozymes. It is reported that the *Ch*-type lysozymes display a broad antimicrobial spectrum, even inhibited the growth of several lactic acid bacteria including *L. delbrueckii*, *L. brevis*, *L. lactis*, and *E. faecalis* (Rau et al. 2000). The lytic spectrum of Lysdb was in accordance with that of *Ch*-type lysozyme. Lysdb also lysed the food spoilage bacteria (Fig. 6), suggesting Lysdb could be further developed as a biopreservative in food processes.

Commonly, endolysins are applied as an exogenous supplement (Rodríguez-Rubio et al. 2015). Therefore, mass production of endolysin is needed. However, the formation of inclusion bodies, the subsequent purification process and the bacterial endotoxin make it a challenge to produce active and food-safe endolysin from *E. coli* cells in large quantities (Fischetti 2010). Fortunately, Lysdb could not lyse *L. lactis* CCTCCAB20102111 and *L. casei* BL23 (Fig. 6). *L. casei* is one of the most prominent non-starter lactic acid bacterial (NSLAB) species found in ripening Cheddar cheese (Settanni and Moschetti 2010). Therefore, we chose *L. casei* as the host cell for the delivery of Lysdb under a modified NICE system.

To evaluate the availability of Lysdb as an antibacterial agent, a lab-scale model cheese was conducted using the engineered *L. casei* BL/pBLysdb and *L. lactis* CCTCCAB20102111 as starters as described previously (Xu and Kong 2013). Our results indicated that *L. casei* BL/pBLysdb was able to kill *S. aureus* in situ, with the final viable count of *S. aureus* reduced by  $10^5$ -fold compared with the raw milk at the end of fermentation. Interestingly, the final survival

of *S. aureus* in the control cheese (*L. lactis* and *L. casei* BL/pB6) was also  $10^2$ -fold lower than that in the raw milk, indicating that the acidic environment also inhibited the growth of *S. aureus* to a certain extent. Other reports have also demonstrated that the production of lactic acid by lactobacilli is an important natural antimicrobial (Gervasi et al. 2014b; Leberer et al. 2008).

Phage endolysins are supposed to be one-use enzymes with a short half-life (Fischetti 2008). Therefore, the use of a protein as a preservative might raise a question about long-term control in the food industry, because the growth of *S. aureus* was hard to standstill, even though the initial viable counts were undetectable (Delbes et al. 2006; Mayer et al. 2012). In our model cheese, the viable numbers of *S. aureus* continuously decreased and remained at a low level of  $10^4$  CFU/g at the end of 6 weeks' cheese ripening, indicating that the Lysdb delivered by *L. casei* was effective throughout the ripening processes. Therefore, the use of engineered endolysin-delivering *L. casei* strain might facilitate the long-term bio-control of *S. aureus* during cheese manufacture from raw milk.

In conclusion, the results presented in this study clearly showed that the *Lactobacillus* phage endolysin Lysdb possesses a muramidase domain similar to *Ch*-type lysozymes, which was able to lyse the live cells of *S. aureus*, as well as other food-borne spoilage bacteria. Active Lysdb delivered by the food grade host *L. casei* could efficiently combat *S. aureus* contamination during cheese making from raw milk. Taken together, the results indicated that Lysdb had the potential to be developed as an effective tool to treat spoilage bacteria during food production processes.

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### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflict of interest.

**Human and animal rights and informed consent** This article does not contain any studies with human participants or animals performed by any of the authors.

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