Construction and characterization of thymidine auxotrophic (ΔthyA) recombinant Lactobacillus casei expressing bovine lactoferricin

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Abstract

Background: Lactobacillus casei (L. casei) is well known for its probiotic property in human and animals. Lactoferricin (Lfcin) polypeptide can effectively modulate host immune responses and have antimicrobial activity in vivo and in vitro. In order to develop a food-grade L. casei system constitutively expressing bovine Lfcin, this study constructed a thymidine auxotrophy (ΔthyA) recombinant L. casei.

Results: Based on the thymidylate synthase gene (thyA) insert site, LFEC (Lfcin expression cassette) was inserted into L. casei genome through homologous recombination, successfully expressed and could be stably inherited. The recombinant L. casei, ΔthyA L. casei-LFEC, is sensitive to chloramphenicol and limited when cultured without thymine. Meanwhile, ΔthyA L. casei-LFEC has both good antibacterial activity against Escherichia coli and Staphylococcus aureus and antiviral activity against porcine epidemic diarrhea virus (PEDV).

Conclusions: We successfully constructed a recombinant L. casei strain expressing Lfcin, ΔthyA L. casei-LFEC, which could only survive in the presence of thymine, and had excellent antimicrobial and antiviral activity with good genetic stability and sensitivity. This research provides a cost-effective alternative to the antibiotics with additional biological functions and wider applicability prospect. Using ΔthyA as the selectable mark instead of antibiotic to construct genetic engineering L. casei provides a safe and effective approach of feed additives in livestock raising.

Keywords: Recombinant Lactobacillus casei, Thymidine auxotrophy, Expression of Lfcin

Background

Lactobacillus casei (L. casei) is well known as a kind of gram positive bacteria with its probiotic property for human and animals, which can maintain microflora homeostasis, inhibit pathogens growing and regulate pH balance in the host gastrointestinal environment [1]. Compared to Escherichia coli (E. coli) expression system, the most significant advantage of L. casei expression system is that the genetic engineered L. casei with vaccine and pharmaceutics purposes can be directly applied via oral administration [2–4]. However, antibiotic resistances are common used as selectable mark for the construction of genetic engineering L. casei expression systems [5], which would result in potential risk to the environment and human.

Thymidylate synthase encoded by thymidylate synthase gene (thyA) is a kind of highly conserved isozyme present in different bacteria, which plays a crucial role in DNA synthesis, and works as the key enzyme in de novo synthesis of phosphorylated deoxythymidine uracil (dTMP) by catalyzing deoxyuridine ribonucleotides (dUMP) into dTMP via methylation modification [5, 6]. When the thyA is deleted, the DNA de novo synthesis pathway in L. casei will be blocked, resulting in proliferation failure [7]. Meanwhile, addition with thymidine or thymine in culture as
the substrate for dTMP synthesis may promote the growth of *L. casei* [5].

Lactoferricin (Lfcin) polypeptide is dissociated off from lactoferrin under acidic condition, which can effectively modulate host immune responses, such as recruiting and promoting the balance of the production of immune cells [8]. The Lfcin has antimicrobial activity in vivo and in vitro [9]. Lfcin with positive charges could establish nonspecific binding with the lipid layer carrying negative charges in cell walls, and then induce autolysis death of bacteria cell by increasing its membrane permeability [10]. Moreover, the Lfcin could block the iron intake of microorganisms to act antimicrobial activity [11]. The Lfcin also possesses many other probiotic properties, such as antioxidation, antiviral activity, inhibiting tumor cell growth and regulating the immunity of the organism [12–16].

In this study, we successfully constructed a genetic engineering *L. casei* using ΔthyA as the selectable mark instead of antibiotic, followed by the expression of bovine Lfcin as a multifunctional protein, suggesting a safe and effective approach for feed additives of livestock or in other industries.

**Methods**

**Bacterial strains, cell strain and virus strain**

Bacterial strains used in this study are listed in Table 1. *L. casei* and ΔthyA *L. casei* was cultured in GM17 broth supplemented with 40 μM of thymidine at 37°C. The temperature-sensitive plasmid pGBHCupp was constructed in our laboratory, containing a pWV01 replicon and chloramphenicol resistance genes. VERO cell line (ATCC® CCL-81™, USA) was stored in our laboratory. PEDV LJB/03 was preserved in our laboratory at −80°C.

**Construction of the pGBHCupp-TF-LFEC-TR**

The HCE promoter, T7 g10 enhancer, signal peptide of peptidoglycanhydrolase and Myc tag, and the two segments of bovine Lfcin (LfcinB and Lfampin) connected with linker were synthesized and inserted into pUC57 vector by Geneewiz Biological Technology Company, Ltd., Beijing, China. The recombinant plasmid was named pUC57–Lfcin. The terminator rrnBT1T2 gene was amplified by PCR using pMD18-T-HCE-MCS-rrnBT1T2 plasmid DNA as template. The rrnBT1T2 fragment was gel purified, inserted into pMD19-T-vector (Takara, DaLian, China), and named pMD19-TS-rrnBT1T2. To construct LFEC(Lfcin expression cassette), Lfcin was digested off from pUC57–Lfcin and inserted into pMD19-TS-rrnBT1T2 (Fig. 1a). The recombinant plasmid was named pMD19-TS-LFEC.

For homologous recombination in *L. casei* 393, upstream homologous arm TF and downstream homologous arm TR were PCR amplified using *L. casei* 393 genomic DNA as a template to create an internal deletion in the thyA site. The two fragments were gel purified, inserted into the pMD19-T vector and named pMD19-TS-TFTR. Then, the LFEC was digested off from pMD19-TS-LFEC, inserted into pMD19-TS-TFTR and named pMD19-TS-TF-TFEC-TR.

The TF-LFEC-TR was digested off from pMD19-TS-TF-LFEC-TR, inserted into pGBHCupp vector (Fig. 1c) and named pGBHCupp-TF-LFEC-TR, which was both temperature sensitive and chloramphenicol resistant.

**PCR primers used for amplifying target genes are listed in Table 2.**

**Electrotransformation**

Briefly, 100 ml culture of *L. casei* cells was grown in MRS medium at 37°C for 4 h. The cells were ice-bathed

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

| Strain          | Genotype / characteristics                                      |
|-----------------|----------------------------------------------------------------|
| E. coli strains | E. coli cloning host                                            |
| TG1             |                                                               |
| pMD18-T-HCE-MCS-rrnBT1T2/TG1 | Tg1 harboring pMD18-T-HCE-MCS-rrnBT1T2, Cmr'                    |
| pGBHC-Pupp/TG1  | Tg1 harboring pGBHC-Pupp, Cmr'                                  |
| pUC57-Lfcin/JM109 | JM109 harboring pUC57-Lfcin, Am' (Synthesis by Geneewiz Biological Technology Co., Ltd) |
| pGBHCupp-TF-LFEC-TR/TG1 | Tg1 harboring pGBHCupp-TF-LFEC-TR, Cmr'                      |
| L. casei strains |                                                               |
| ΔuppL. caseiATCC393 | L. casei ATCC393 derivative without upp                        |
| pGBHCupp-TF-LFEC-TR/L.casei ΔuppL. caseiATCC393 harboring pGBHCupp-TF-LFEC-TR, Cmr' |
| ΔthyAL. casei-LFEC | ΔthyAL. casei ATCC393 with Lfcin expression cassette insertion in the thyA |
| Plasmids        |                                                               |
| pGBHC-upp       | Ori (pWV01) with a copy of upp expression cassette, Cmr'          |
| pGBHCupp-TF-LFEC-TR | pGBHC-upp containing Lfcin expression cassette between upstream and downstream sequence flanking the thyA integration site |
Table 2  Primers used in this study

| Primer   | Primer sequence (5'→3') | Product size |
|----------|-------------------------|--------------|
| Cloning primers |
| rmBT1T2  | F: ACTAGTGTCAATGATGACGAGATCTGGCGCTTGGTCGAGATGAGAGAAGAGTTTGTAGAAACGCAAAAGGC R: CTGGAGAGAGTTTGTAGAAACGCAAAAGGC | 438 bp |
| LFEC     | F: TCTAGAGATCTCTCCTTCACAGATTCCCAATGAGAGAAGAGTTTGTAGAAACGCAAAAGGC R: CTGGAGAGAGTTTGTAGAAACGCAAAAGGC | 1022 bp |
| TF       | F: GCCGCCGCTTTAGCGACGACCGTTCATGAGAGAAGAGTTTGTAGAAACGCAAAAGGC R: TTAATTAAGGGTCACGAGCAAGGTAT | 1020 bp |
| TR       | F: ACTAGTGTCAATGATGACGAGATCTGGCGCTTGGTCGAGATGAGAGAAGAGTTTGTAGAAACGCAAAAGGC R: CTGGAGAGAGTTTGTAGAAACGCAAAAGGC | 1392 bp |
| Screening primers |
| thyA     | F: TGTGGCATCACTTAGGACC R: TGTGGCATCACTTAGGACC | 3268 bp |
| Real-time PCR primes |
| PEDV-N   | F: ACTGAGGCTGTCCGCTTGGACGAGATGAGAGAAGAGTTTGTAGAAACGCAAAAGGC R: GGGTCAACAATCTCACAATACATGG | 137 bp |
| Beta-actin | F: AAGGATTACATATGTTGGCGATG R: TCTCAATGCTCGTCCCAGTGGT | 103 bp |

Enzyme restriction sites are underlined.
for 10 min, harvested by centrifugation at 5000 g for 10 min at 4°C, and resuspended in 20 ml of ice-cold EPWB buffer. Then, the cells were harvested and resuspended in ice-cold EPWB buffer for two more times as described above. Finally, the competent cells were harvested and resuspended in 1 ml EPB buffer. 200 ng of pGBHCupp-TF-LFEC-TR plasmid was added to 200 µl of competent cells in ice bath and transformed by electroporation at 2.0 kV. Cells were recovered in MRS medium for 2–3 h and then spread on MRS agar plate containing 2.5 µg/ml of chloramphenicol. The positive colony was identified and the recombinant pGBHCupp-TF-LFEC-TR/L. casei was obtained.

**Homologous recombination of L. casei ATCC 393**

The process of homologous recombination was followed with temperature sensitive selection (Fig. 1b). The integration transformants were transferred (1% inoculum) three times at 43°C and grown to stationary phase each time to select single-crossover integrations, after which the pGBHCupp vector containing chloramphenicol resistant gene stopped replication. Then, the single-crossover integrations (1% inoculums) were propagated in GM17 broth for 30 generations in the absence of antibiotic resistance to achieve the second homologous crossover and lose the recombinant plasmid. The presence of the LFEC insertion in ΔthyA L. casei chromosome was detected by PCR with the primers thyA F and thyA R shown in Table 2. The positive integration L. casei was named as ΔthyA L. casei-LFEC.

**Test of auxotrophy and chloramphenicol-sensitivity**

In order to test the dependence of ΔthyA L. casei-LFEC on thymine, the ΔthyA L. casei-LFEC was cultured overnight in GM17 broth supplemented with thymine and spread on GM17 agar plates in the presence or absence of thymine for incubation at 37°C for 48 h. Then, the bacteria concentration was determined. In parallel, L. casei was used as control. In order to detect plasmids residual in ΔthyA L. casei-LFEC for chloramphenicol resistance, the bacteria cultured overnight in MRS broth supplemented with thymine was spread on MRS agar plates in the presence or absence of chloramphenicol at 37°C for 48 h to observe.

**Growth kinetics of the ΔthyA L. casei-LFEC**

In order to analyze growth kinetics of the ΔthyA L. casei-LFEC, the growth curve of the ΔthyA L. casei-LFEC was determined in the presence or absence of thymine. The bacteria density was measured by absorbance at OD600 every 2 h until 48 h. In parallel, L. casei cultured in GM17 broth in the presence or absence of thymine was used as control.

**Stability of the ΔthyA L. casei-LFEC**

To determine the recombinant strain stability post chromosomal integration, the culture of ΔthyA L. casei-LFEC (1% inoculum) was continuously transferred 50 generations at interval of 12 h, then genomic DNA of each generation was extracted and detected by PCR for the presence of LFEC. PCR primers used in this study are listed in Table 2.

**Determination of Lfcin expressed by ΔthyA L. casei-LFEC**

Western blot assay was performed to analyze the expression of Lfcin in ΔthyA L. casei-LFEC cultured in GM17 broth supplemented with thymine. Following the extraction of total proteins in 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were electro-transferred onto a nitrocellulose membrane, incubated with mouse anti-LfcinB antibody prepared in our lab and horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG antibody diluted at 1:5000 (Invitrogen, USA), and visualized with a chemiluminescent substrate reagent according to the manufacturer’s instruction [17, 18].

Indirect immunofluorescence assay was performed to detect the expression of Lfcin on the cell surface of ΔthyA L. casei-LFEC as previously described [19]. Briefly, 1 mL of ΔthyA L. casei-LFEC cultured in GM17 broth for 12 h was centrifuged, washed with PBS three times, and resuspended in 1 mL of sterile PBS-3% bovine serum albumin (BSA) containing mouse anti-myc antibody; following the incubation at 37°C for 1 h, the cells were harvested, washed three times, and incubated in 1 mL of FITC-conjugated goat anti-mouse IgG antibody (diluted at 1:500) at 37°C for 1 h; then, the cells were washed three times, transferred onto a glass slide, and fixed with cold acetone for 30 min; Confocal microscope was used to observe fluorescence signals. In parallel, L. casei was used as negative control.

The Lfcin concentration in the supernatant of ΔthyA L. casei-LFEC was determined using the native bovine Lfcin (XingHao Pharmaceutical co., ltd., WuHan, China) as standard sample and anti-Lfcin monoclonal antibody (preserved in our lab) as detection antibody.

**Antimicrobial activity test of Lfcin**

To determine the antimicrobial activity of Lfcin, single bacterial colony of E. coli and S. aureus were inoculated in LB broth respectively, then 10 mL of filtered (0.22 µm) supernatant of ΔthyA L. casei-LFEC was added when the OD600 value reached 0.3–0.4. In comparison, the supernatant of L. casei culture was used as negative control. Bacteria concentration of each group was detected by determining OD600 value at intervals of 2 h. At every time point, the colony counting of E. coli and S.
Transmission electron microscopy (TEM)
To study the insight of the direct effects of Lfcin in the morphology of bacterial cells, logarithmic growth phase of S. aureus and E. coli cells after treatment with the supernatant of ΔthyA L.casei-LFEC were chosen to assess the bacterial membrane damage by TEM. After incubation, the cells were pelleted by centrifugation at 1000 rpm for 5 min, followed by washing thrice with PBS. Subsequently, the cells were fixed with 2.5% glutaraldehyde for 1 h, washed in PBS for three times, centrifuged in a series of increasing ethanol (30, 50, 70, 90% and absolute ethanol) 20–25 min [20]. The cells were penetrated with acetone and embedding agent at a 1:1 volume, shaken for 2 h by an oscillator (Qilinbeier, Jiangsu, China), and again shaken for 2 h in the pure embedding agent before polymerization in the incubator at 37°C for 24 h, 45°C for 48 h, and 60°C for 48 h. Next, 120 nm ultra-thin slices were sectioned and stained with 4% uranyl acetate for 20 min and with double electron staining with lead citrate for 5 min. These ultra-thin sections were then placed on a single-hole copper mesh and were subjected to observation and photography under electron microscopy.

Antiviral activity test of Lfcin
VERO cells were grown in 96-wells tissue culture plates at 37°C in 5% CO₂ until 85% confluence and infected with PEDV at 1.0 MOI for 1 h at 37°C [21]. For the experiment groups, 100 μL filtered supernatant of ΔthyA L.casei-LFEC was added to VERO cells before PEDV absorption, simultaneously with PEDV and after PEDV absorption. For the control groups, 5.0 ng/L of native bovine Lfcin was added as positive control, while the filtered supernatant of L.casei was added as negative control. VERO cells were frozen and thawed for three times at 72 hpi, after which total RNA was extracted using Fast2000 RNA kit and cDNA was gained using Reverse Transcription enzyme (Toyobo, Japan). Real-time fluorescent quantitative PCR was performed to detect the viral replication using FastStart Universal SYBR Green Master (Roche, Switzerland) and ABI 7500 real time PCR system was used to determine the viral replication.

Statistical analysis
The results were analyzed as the (1/2−ΔΔCt) ± SD [21], theΔΔCt = (CtPEDV N gene−Ctβ-actin) Lfcin/(CtPEDV N gene−Ctβ-actin)negative control. Comparisons between groups were performed using analysis of Tukey’s. The P value of <0.05 (P < 0.05) was considered as statistically significant and P < 0.01 as highly significant.

Results
Construction of the ΔthyA L.casei-LFEC
LFEC was inserted between the homologous arms (TF and TR) as indicated in Fig. 1b. As confirmed by PCR results (Fig. 2), following the construction of pGBHC-upp-TF-LFEC-TR/L.casei, the LFEC constitutively expressing Lfcin was successfully inserted into L.casei genome via homologous recombination.

Test of auxotrophy and chloramphenicol-sensitivity of ΔthyA L.casei-LFEC
The results showed that only pGBHCup-TF-LFEC-TR/L.casei (PT) could grow on the GM17 plate supplemented with Cmr and thymine (Fig. 3a), indicating no residual antibiotic resistance present in ΔthyA L.casei-LFEC; on the GM17 plate supplemented with thymine and without Cmr, L. casei, pGBHCup-TF-LFEC-TR/L.casei and ΔthyA L.casei-LFEC could normally grow (Fig. 3b); on the GM17 plate without thymine and Cmr, L. casei and pGBHCup-TF-LFEC-TR/L.casei could normally grow, but not ΔthyA L.casei-LFEC (Fig. 3c). Our results indicated that the ΔthyA L.casei-LFEC without Cmr resistance was constructed successfully.

Determination of growth kinetics of ΔthyA L.casei-LFEC
Compared to wild type L. casei, ΔthyA L.casei-LFEC could grow normally with the addition of thymine in GM17 broth. However, the growth of ΔthyA L.casei-LFEC was limited in the absence of thymine,
indicating the thymine dependence of ΔthyA L.casei-LFEC growth (Fig. 4).

**Genetic stability of ΔthyA L.casei-LFEC**

The ΔthyA L.casei-LFEC was serially cultured for 50 generations and the presence of the LFEC integrant was detected by PCR. As shown in Fig. 5, the LFEC was still detectable in the thyA location of ΔthyA L.casei-LFEC, indicating good genetic stability.

**Expression of Lfcin by ΔthyA L.casei-LFEC**

Western blot analysis of Lfcin expression showed an immunoblot band of expected size (Fig. 6a), while indirect immunofluorescence results showed obvious
fluorescence on the cell surface of ΔthyA L. casei-LFEC (Fig. 6b). Both experiments confirmed the successful expression of Lfcin in ΔthyA L. casei-LFEC. The Lfcin concentration in the supernatant of ΔthyA L. casei-LFEC was determined to be 23.37 μg/mL.

**Antibacterial activity of Lfcin expressed by ΔthyA L. casei-LFEC**

Compared to wild type L. casei, the growth of both S. aureus and E. coli were obviously limited and the inhibition percentage were 42.22 and 40.05% higher respectively when cultured with Lfcin, indicating a strong antibacterial activity of Lfcin (Fig. 7). Meanwhile, TEM results showed that Lfcin lead to damages in cell morphology of S. aureus and E. coli, including rough cell surface and cell lysis (Fig. 8b, d, f and h), which indicated the potential bactericidal effect of Lfcin.

**Antiviral activity of Lfcin expressed by ΔthyA L. casei-LFEC**

The effect of Lfcin on the cellular receptors and viral proteins was tested with simultaneous incubation conditions. A significant inhibition of viral replication was observed when native bovine Lfcin or Lfcin expressed by ΔthyA L. casei-LFEC were added with viral together, compared to the negative control (Fig. 9).

**Discussion**

Using genetic engineered lactic acid bacteria (LAB) to deliver functional protein is a promising approach especially for oral administration development, which could effectively protect protein from protease digestion and induce effective functions [22–25]. LAB expression system has been widely used in oral vaccine development, whose effect has been proven both in vitro and in vivo. Anbazhagan et al. generated a recombinant LAB that constitutively overexpressed B. subtilis oxalate decarboxylase and degraded oxalate efficiently under in vitro conditions [26]. Moreover, Giselli et al. constructed a recombinant LAB that could secrete attenuated recombinant staphylococcal enterotoxin B and induce a protective immune response in a murine model of S. aureus infection [27]. Currently, for the construction of LAB expression system, some antibiotic resistances are widely used as selection marker. However, with the application of genetic engineered LAB harboring, the
Fig. 7 Antibacterial activity analysis of Lfcin. (a) S. aureus, growth curve of S. aureus; Negative control, growth curve of S. aureus cultured with L. casei filtered supernatant; Lfcin, growth curve of S. aureus cultured with ΔthyA L. casei-LFEC filtered supernatant; (b) E. coli, growth curve of E. coli; Negative control, growth curve of E. coli cultured with L. casei filtered supernatant; Lfcin, growth curve of E. coli cultured with ΔthyA L. casei-LFEC filtered supernatant; (c) Inhibition percentage of S. aureus cultured with ΔthyA L. casei-LFEC filtered supernatant; (d) Inhibition percentage of E. coli cultured with ΔthyA L. casei-LFEC filtered supernatant.

Fig. 8 Transmission electron microscopy display of the antibacterial activity of Lfcin. (a, c) Untreated S. aureus; (b, d) S. aureus treated with Lfcin; (e, g) Untreated E. coli; (f, h) E. coli treated with Lfcin.
Antibiotic resistance genes would be exposed to the environment and animals, causing potential risk to human.

In this study, we successfully constructed a thyA-based auxotrophy genetic engineered L. casei (ΔthyA L. casei-LFEC) with the deletion of antibiotic resistance gene as selection marker. The growth of thyA auxotrophy L. casei is high limited by the additional adding thymine or thymidine in the culture media, showing high thymine dependence. Compared to the antibiotic resistance selection marker, the most significant advantage of auxotroph selection marker is the safety to the environments. Thus, the ΔthyA L. casei-LFEC constructed in this study would provide a promising approach for food-grade agents’ development.

Lfcin is a kind of polypeptide with diverse biological functions, such as antibacterial effect, antiviral activity, antitumor effect and immunity modulation, suggesting a potential agent for food additive or immunologic adjuvant [28]. Bovine Lfcin was usually produced in vitro by yeast or E. coli [29, 30], while the complex purification process is required. Using lactic acid bacteriato deliver it suggested an alternative approach for utilizing Lfcin via oral administration. In the present study, by using the ΔthyA L. casei as delivery carrier and the Lfcin as the target functional protein, a genetic engineered L. casei constitutively expressing Lfcin, ΔthyA L. casei-LFEC, was constructed through two-step homologous recombination. The recombinant ΔthyA L. casei-LFEC was passed for 50 generations and showed good genetic stability. In our previous study, wild-type L. casei has already been applied to construct chromosomal insertion strains using homologous recombination which showed similar genetic stability to this study [25].

Furthermore, the antimicrobial activity and the antiviral activity of the Lfcin were evaluated in vitro. The growth curve results of E. coli and S. aureus indicated that the growth of both pathogenic bacteria were significantly inhibited when cultured with Lfcin. Also, E. coli and S. aureus showed cell damages in morphology in TEM experiment, which confirmed the antibacterial activity of Lfcin expressed in ΔthyA L. casei-LFEC. Meanwhile, TEM results showed that Lfcin caused changes in cell membrane permeability which lead to nucleic acid area leak and the loss of bacteria pathogenesis. In addition, the antiviral effect of Lfcin on PEDV replication was evaluated by determining the inhibition fold-change of PEDV replication in three different infection phases treated with Lfcin. Lfcin showed significantly stronger inhibition effect than negative control in the group of Lfcin treatment pre-PEDV absorption, while there was no significant difference between Lfcin and negative control in the group of simultaneous addition of Lfcin with PEDV, indicating a possibility that Lfcin might affect the susceptibility of the cell receptors to PEDV, and the affinity of PEDV with cell receptors had impact on the inhibition effect of Lfcin. It has also been reported in other studies that the block of the binding between PEDV and the cellular receptors could effectively reduce PEDV infection [31–33]. Interestingly, in the group of Lfcin treatment after-PEDV absorption, whose overall inhibition effect of three different infection phases was obviously lower than that of the other two groups, Lfcin still showed a significantly stronger inhibition effect than negative control. We speculated that Lfcin might activate a different cell defense mechanism after failing to block PEDV at the phase of viral infection.
entry, which needs further confirmation. As negative control, the supernatant of *Lactobacillus casei* also showed inhibitory effect on PEDV replication, indicating unspecific antiviral activity of *Lactobacillus casei* which has been also proven in other reports [34–39].

**Conclusion**

In summary, we constructed a genetic engineered auxotrophy *L. casei* expressing Lfcin (*AtlytA L.casei*), which has antibacterial and antiviral activities. This research provides a safe and effective approach for oral functional protein and other pharmacutes purposes.

**Abbreviations**

Cmr: Chloramphenicol; dTMP: Deoxythymidine uracil; dUMP: Deoxyuridine acid bacteria; Lfcin: Lactoferricin; PCR: Polymerase chain reaction; PEDV: Porcine epidemic diarrhea virus; SD: Standard deviation; SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TEM: Transmission electron microscopy; VERO: Verda Reno; ΔAtlytA: Thymidine auxotrophy

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**Availability of data and materials**

The data analyzed during the current study are available from the corresponding author on reasonable request.

**Authors’ contributions**

Dr. LT had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. Study concept and design: LT, YL and YX. Experiment performance: XL, ZW, HT and LW. Analysis and interpretation of the data: XL, ZW, HT and LW. Drafting of the manuscript: HZ and XL. Critical revision of the manuscript for important intellectual content: All authors. Statistical analysis: XL, XQ, YJ. Study supervision: LT, YL and YX. All authors read and approved the final manuscript.

**Ethics approval**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

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