

Synergistic therapeutic effect of isochlorogenic acid (ICGA) combined with the bacteriophage lyase Lys40 against Salmonella-induced mastitis: In vitro and in vivo evaluations

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Case Report

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Title: Synergistic therapeutic effect of isochlorogenic acid (ICGA) combined with the bacteriophage lyase Lys40 against *Salmonella*-induced mastitis: *In vitro* and *in vivo* evaluations

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Abstract

Background: Bovine mastitis caused by *Salmonella* spp. presents a significant challenge to the dairy industry because of increasing antimicrobial resistance (AMR) and the risk of antibiotic residues in milk. Bacteriophage-derived endolysins are promising alternatives, but their efficacy against gram-negative bacteria is often limited by the outer membrane (OM) barrier. Isochlorogenic acid (ICGA), a plant-derived polyphenol, has membrane-permeabilizing properties. This study investigated the synergistic antibacterial activity of ICGA combined with the recombinant phage lyase Lys40 against *Salmonella* and its therapeutic potential in mastitis models.

Methods: The synergistic effect was evaluated via the checkerboard method to determine the fractional inhibitory concentration index (FICI). The antibacterial spectrum was assessed against 24 mastitis-associated isolates. *In vitro* efficacy was determined via the use of a *Salmonella*-infected bovine mammary epithelial cell (bMEC) model, in which cell viability (CCK-8), lactate dehydrogenase (LDH) release, bacterial adhesion/invasion, and cellular morphology were analyzed. *In*

vivo therapeutic effects were evaluated in a *Salmonella*-induced murine mastitis model via histopathology, bacterial load quantification, and immunohistochemical analysis of apoptotic markers (Caspase-3, Caspase-9, and Cyt-c).

Results: Lys40 alone showed weak activity against *Salmonella* CVCC-529 (MIC > 1 mg/mL). However, combination with ICGA significantly reduced the MIC of Lys40 to 0.200 mg/mL (FICI = 0.45), indicating strong synergy, particularly at pH 8–9. The combination demonstrated broad-spectrum activity against both gram-negative (e.g., *E. coli* and *Salmonella*) and gram-positive pathogens (e.g., *S. aureus*). In bMECs, the combination significantly inhibited bacterial adhesion and invasion ($p < 0.05$), reduced LDH release, and preserved cellular morphology. Compared with the untreated infection, the combined treatment significantly decreased the mammary tissue bacterial load and histopathological damage in the murine model ($p < 0.01$). Furthermore, the treatment downregulated the expression of proapoptotic proteins (caspase-3, caspase-9, and Cyt-c), protecting mammary tissue from apoptosis.

Conclusions: The combination of ICGA and Lys40 exerts a potent synergistic effect by overcoming the outer membrane barrier, suggesting a promising antibiotic-free therapeutic strategy for controlling *Salmonella*-induced mastitis.

Keywords: Mastitis; *Salmonella*; Phage lyase; Isochlorogenic acid; Synergism; Apoptosis.

1. Introduction

Bovine mastitis remains one of the most prevalent and costly diseases in the dairy industry worldwide, causing significant economic losses through reduced milk yield and quality^[1]. While gram-positive bacteria are traditional causative agents, gram-negative pathogens, including *Salmonella* spp. and *Escherichia coli*, are increasingly isolated from clinical and subclinical cases^[2]. *Salmonella* infections are particularly concerning because of their potential for zoonotic transmission via milk and the rapid emergence of multidrug-resistant (MDR) strains^[3].

The conventional reliance on antibiotics for mastitis therapy has accelerated the development of AMR and raised public health concerns regarding drug residues in dairy products^[4]. Consequently, novel, nonantibiotic therapeutic strategies are urgently needed. Bacteriophage-derived endolysins (lysins) have emerged as a potent class of enzybiotics^[5]. They function by hydrolyzing the peptidoglycan layer of the bacterial cell wall, causing rapid lysis. However, a major li

mitation of the use of exogenous lysins against gram-negative bacteria is the presence of the outer membrane (OM), which restricts access to the peptidoglycan layer^[6].

To overcome this barrier, "arstilysin" strategies or combination therapies utilizing OM permeabilizers (OMPs) have been proposed. Isochlorogenic acid (ICGA), a naturally occurring polyphenol found in plants such as *Lonicera japonica* (Honeysuckle) and coffee, has antioxidant, anti-inflammatory, and broad-spectrum antimicrobial properties^[7]. Previous studies have suggested that certain polyphenols can interact with the lipid A component of lipopolysaccharides (LPS) and chelate divalent cations, thereby destabilizing the gram-negative OM^[8].

We hypothesized that ICGA could act as a permeabilizer to increase the lytic activity of the recombinant phage lyase Lys40 against gram-negative mastitis pathogens. This study aims to (1) determine the synergistic antibacterial activity and optimal conditions for the ICGA-Lys40 combination; (2) evaluate its protective effects on bovine mammary epithelial cells (bMECs) *in vitro*; and (3) assess its therapeutic efficacy and mechanism of action, specifically with respect to tissue apoptosis, in a *Salmonella*-induced murine mastitis model.

2. Materials and methods

2.1 Bacterial strains and chemicals

Salmonella CVCC-529, *Escherichia coli* ATCC-25922, and *Staphylococcus aureus* ATCC-29213 were purchased from the China Institute of Veterinary Drug Control. Clinical isolates (including *E. coli*, *S. aureus*, *Streptococcus*, etc.) were preserved in our laboratory. Isochlorogenic acid A (ICGA) was provided by Chenguang Biotech Group. The lysin Lys40 was expressed in our laboratory and purified via a nickel-chelating TED column system.

2.2 Determination of Synergistic Activity

The minimum inhibitory concentrations (MICs) of ICGA and Lys40 against *Salmonella* CVCC-529 were determined via the microbroth dilution method. Different concentration combinations were designed via the microtiter checkerboard method (Table 1), and the fractional inhibitory concentration index (FICI) was calculated according to the following formula:

$$FICI = \frac{MIC_{A(combined)}}{MIC_{A(alone)}} + \frac{MIC_{B(combined)}}{MIC_{B(alone)}}$$

On the basis of the calculated FICI value, the interaction between the two dru

gs was interpreted as follows: synergy when $FICI \leq 0.5$; additive effect when $0.5 < FICI \leq 1$; indifference when $1 < FICI \leq 2$; and antagonism when $FICI > 2$.

Table 1 Grouping and processing of the microchysboard method

ICGA Concentration (mg/mL)	Lys40 lyase concentration (mg/mL)				
	0.000	0.050	0.100	0.150	0.200
25.000	Into each well, 50 μ L of ICGA solution and 50 μ L of Lys40 lyase solution, each at the designated concentration, were added.				
12.500					
6.250					
3.125					
1.563					
0.781					
0.000					

Note: The optical density (OD) of each well was measured via a microplate reader (OD600).

2.3 Determination of Broad-Spectrum Antibacterial Activity

To evaluate the antibacterial spectrum of the combination of ICGA and Lys40 against common pathogens responsible for bovine mastitis, five gram-positive bacterial strains and nineteen gram-negative bacterial strains were selected for testing. These included various serotypes of *Escherichia coli*, *Salmonella*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, and other clinical isolates as well as standard strains.

$$\text{Inhibition Rate (\%)} = \frac{OD_{\text{positive}} - OD_{\text{test}}}{OD_{\text{positive}} - OD_{\text{negative}}} \times 100\%$$

2.4. In vitro Bovine Mammary Epithelial Cell (bMEC) Model

bMECs were cultured in DMEM supplemented with 10% fetal bovine serum.

- **Cytotoxicity & viability:** Cell viability was measured via the CCK-8 assay.
- **Adhesion and Invasion Assays:** bMECs were infected with *Salmonella* (MOI = 10:1 or 100:1) treated with or without the ICGA-Lys40 combination. For invasion, extracellular bacteria were killed with gentamicin (250 μ g/mL). The cells were lysed with 0.5% Triton X-100, and CFUs were counted.
- **LDH release:** Lactate dehydrogenase (LDH) release was measured to quantify cell membrane damage via a commercial kit.
- **Morphology:** The cells were stained with hematoxylin and eosin (H&E) and observed via light microscopy.

2.5. Murine Mastitis Model

The experimental animals were purchased from Nanjing Changjing Biotechnology Co., Ltd

Lactating BALB/c mice (7 days post-partum) were divided into five groups (n=9/group):

1. Control (No infection)
2. PBS (Mock infection)
3. Model (Infection + PBS treatment)
4. Antibiotic Group (Infection + Ceftriaxone Sodium, CRO)
5. Combination Group (Infection + ICGA/Lys40)

Mastitis was induced by intraductal injection of *Salmonella* CVCC-529 (100 μ L, 1.5×10^7 CFU/mL) into the 4th pair of mammary glands. Treatment was administered 24 h post infection via the same route. The mice were euthanized at 24, 36, and 48 h posttreatment.

At the designated time points (24, 36, and 48 h posttreatment), the mice were euthanized via an intraperitoneal injection of an overdose of pentobarbital sodium (150-200 mg/kg). Deep anesthesia and complete loss of consciousness were confirmed by the absence of the pedal withdrawal reflex. Subsequently, cervical dislocation was performed as a secondary physical method to ensure death prior to tissue collection. This method was chosen to minimize animal pain and distress in strict accordance with the AVMA Guidelines for the Euthanasia of Animals, and to avoid artefactual changes in the mammary gland tissues.

2.6. Histopathology and immunohistochemistry (IHC)

Mammary tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned. The sections were stained with H&E for histopathological scoring. For IHC, the sections were incubated with primary antibodies against Caspase-3, Caspase-9, and Cytochrome c (Cyt-c). Protein expression was quantified by measuring the average optical density (AOD) via ImageJ software.

2.7. Statistical analysis

The data are presented as the means $\pm$ standard deviations (SDs). Statistical significance was determined via one-way ANOVA followed by Tukey's post hoc test. $P < 0.05$ was considered statistically significant.

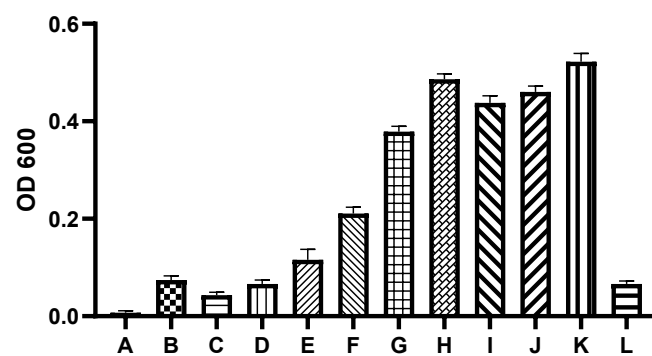
3. Results

3.1 Synergistic Antibacterial Effects of ICGA and Lys40

The MIC of ICGA was 12.500 mg/mL (Fig 1), whereas Lys40 alone exhibited limited activity against *Salmonella* CVCC-529 (Fig 2). However, in the presence of ICGA, the MIC of Lys40 was significantly reduced to 0.200 mg/mL. The checkerboard assay results (Table 2) revealed an FICI of 0.45, indicating strong synergistic interactions between the two agents.

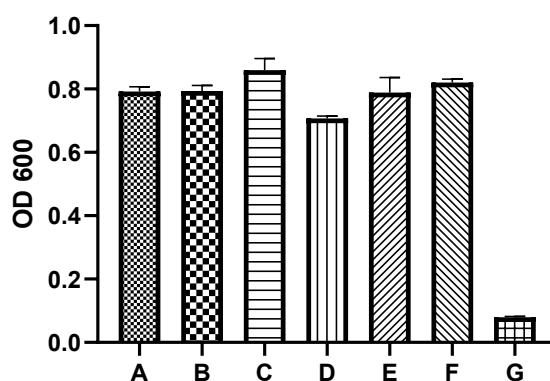
Condition optimization experiments demonstrated that the combination was most effective at pH 8–9 (Fig 3), which aligns with the pathological feature of elevated pH in mastitic milk. The bactericidal curve further revealed that the combination significantly inhibited bacterial growth within 2–8 hours of treatment (Fig 4).

Fig1 Inhibitory effect of ICGA on *Salmonella* CVCC-529



Note: ICGA: A: 100.000 mg/mL; B: 50.000 mg/mL; C: 25.000 mg/mL; D: 12.500 mg/mL; E: 6.250 mg/mL; F: 3.125 mg/mL; G: 1.563 mg/mL; H: 0.781 mg/mL; I: 0.391 mg/mL; J: 0.195 mg/mL; K: Positive control group; L: Negative control group.

Fig 2 Inhibitory effect of the lyase Lys40 on *Salmonella* CVCC-529



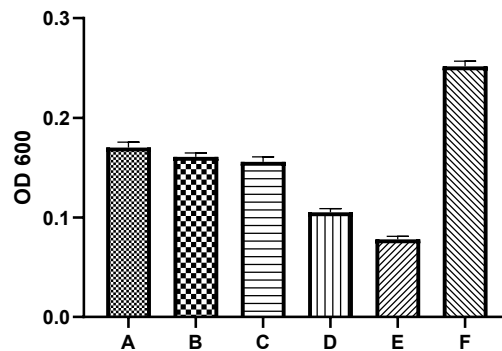
Note: Lys40 lyase concentration: A: 0.0001 mg/mL; B: 0.001 mg/mL; C: 0.01 mg/mL; D: 0.1 mg/mL; E: 1 mg/mL; F: positive control group; G: negative control group.

Table 2 Effects of the combination of lys40 and ICGA on chessboard analysis

ICGA Concentration (mg/mL)	Lys40 lyase concentration (mg/mL)				
	0.000	0.050	0.100	0.150	0.200
25.000	0.07±0.010	0.07±0.006	0.06±0.016	0.03±0.006	0.01±0.005
12.500	0.07±0.007	0.17±0.006	0.19±0.001	0.05±0.010	0.02±0.005
6.250	0.33±0.005	0.32±0.013	0.30±0.006	0.21±0.011	0.14±0.015
3.125	0.49±0.012	0.42±0.009	0.32±0.012	0.20±0.012	0.14±0.015
1.563	0.88±0.021	0.57±0.007	0.47±0.006	0.31±0.004	0.30±0.004
0.781	0.94±0.006	0.87±0.011	0.70±0.012	0.07±0.012	0.59±0.005
0.000	0.96±0.011	0.83±0.006	0.90±0.018	0.88±0.008	0.87±0.022

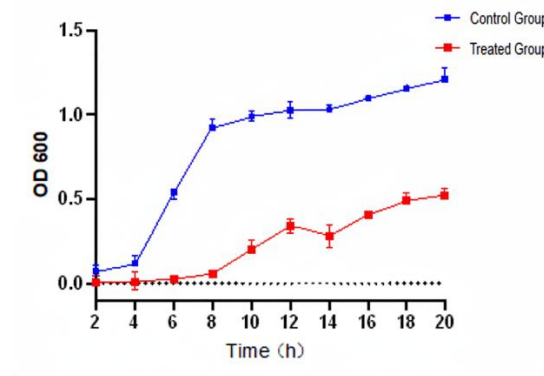
Note: The optical density (OD) of each well was measured via a microplate reader (OD600).

Fig 3 Antibacterial effects of ICGA and lysase Lys40 suspensions at different pH values



Note: ICGA+bacteriophage lysozyme Lys40 (mg/mL): A: pH=5; B: pH=6; C: pH=7; D: pH=8; E: pH=9; F: pH=10

Fig 4 Dynamic time-effect inhibition curves of ICGA and Lys40 lysase suspensions



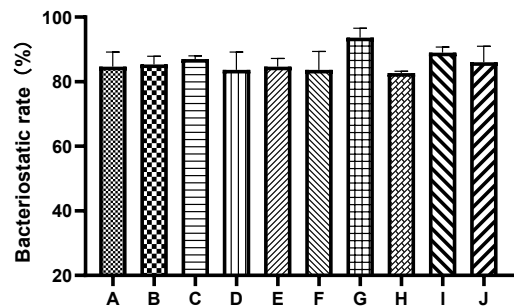
3.2 Broad antimicrobial spectrum

The combination of ICGA and phage lysate Lys40 demonstrated inhibition rates exceeding 80% against different serotypes of *Escherichia coli* (Fig 5). Furthermore, analysis of the inhibition rates against six different *Salmonella* strains (Fig 6) revealed that the combination exhibited high efficacy against both four wild-type *Salmonella* strains and two standard *Salmonella* strains, with all inhibition rates above 80%. For *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*, the inhibition rates all exceeded 80% (Fig 7), indicating favorable antibacterial efficacy.

Streptococcus agalactiae—was performed (Fig 8). The results showed that the combined treatment achieved inhibition rates above 70% for all the tested strains, with a particularly high inhibition rate of $94.67 \pm 3.21\%$ against *Staphylococcus aureus*.

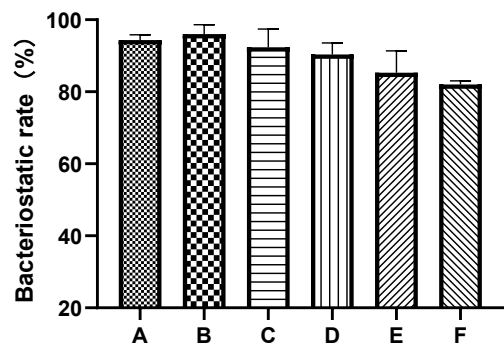
occus aureus, demonstrating favorable antibacterial activity.

Fig 5 Inhibitory effects of ICGA and lys40 suspensions on different serotypes of *Escherichia coli* strains



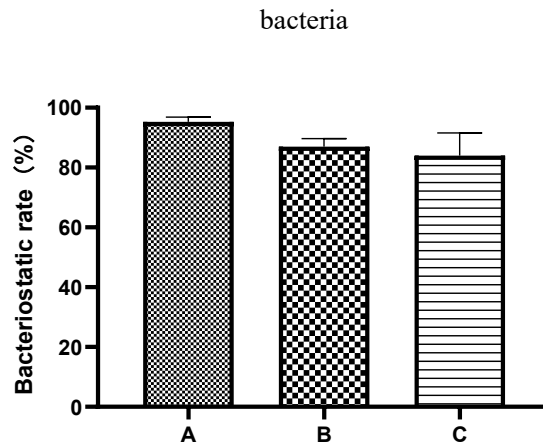
Note: A: ATCC-25922; B: O119; C: O78; D: O2; E: O45; F: O101; G: O25; H: O6; I: O21; J: O9.

Fig 6 Inhibitory effects of ICGA and lys40 suspensions on *Salmonella* strains of different serotypes

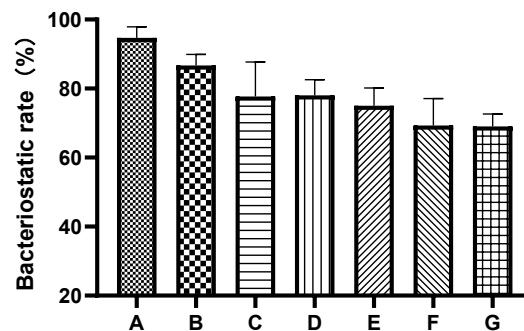


Note: A: Gallinarm; B: Schwarzengund; C: Typhimurium; D: Enteritidis; E: CVCC-529; F: ATCC-13076.

Fig 7Antibacterial effects of ICGA and Lys40 suspensions on several conditional pathogenic



Note: A: *Klebsiella pneumoniae*; B: *Pseudomonas aeruginosa*; C: *Acinetobacter baumannii*
 Fig 8 Antibacterial effects of ICGA and Lys40 suspensions on different gram-positive bacteria



Note: A: *Staphylococcus aureus* ATCC-29213; B: *Bacillus* spp.; C: *Enterococcus faecalis* ATCC-29212; D: *Streptococcus dysgalactiae* ATCC-27957; E: *Streptococcus pneumoniae* ATCC-49619; F: methicillin-resistant *Staphylococcus aureus* (MRSA); H: *Streptococcus agalactiae*.

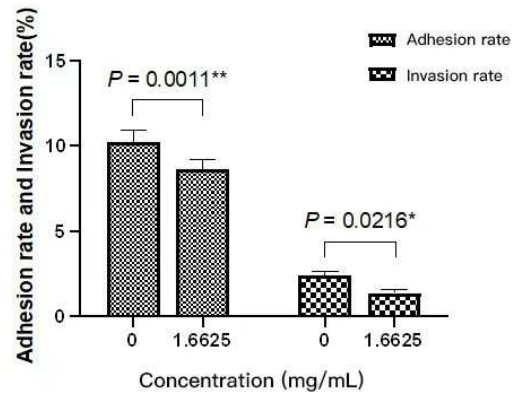
3.3. Protection of bMECs from *Salmonella*-Induced Damage

3.3.1 Effects of combination therapy on the adhesion and invasion rates of bMECs in *Salmonella* infection

As shown in Fig. 9, compared with that of the control group, the adhesion rate of the experimental group was significantly different ($P < 0.01$), and the invasion rate of the experimental group was significantly different ($P < 0.05$). These findings indicate that the suspension of ICGA and the phage lytic enzyme Lys40 has inhibitory effects on the adhesion and invasion of bMECs infected with *Salmonella*.

Fig. 9 Adhesion rate and invasion rate of breast epithelial cells in suspension solution to alleviate

Salmonella infection

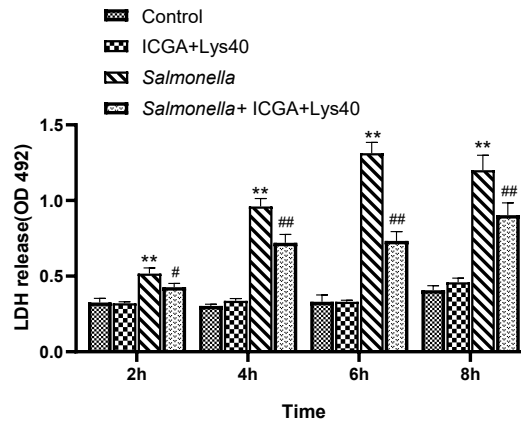


Note: A: Control group; B: Experimental group; * $P < 0.05$; ** $P < 0.01$.

3.3.2 Effects of combination therapy on LDH release from bMECs infected with Salmonella

As shown in Fig. 10, compared with that in the Control1 group, there was no significant change in the release of LDH in the ICGA+Lys40 group after 2 hours of Salmonella infection of bMECs ($P > 0.05$). Although the release of LDH in the Salmonella+ICGA+Lys40 group was greater than that in the control group, it was lower than that in the Salmonella group. At 4 h and 6 h of infection, the release of LDH in the Salmonella group was significantly greater than that in the other experimental groups ($P < 0.01$). Although the release of LDH in the Salmonella+ICGA+Lys40 group was greater than that in the control group, it was significantly lower than that in the Salmonella group ($P < 0.05$). At 8 h of infection, LDH release in the Salmonella group decreased but remained higher than that in the control group, which may be related to prolonged infection leading to massive cell death. These results indicate that the combination of ICGA and the lyase Lys40 can effectively improve the damage caused by Salmonella to bMECs.

Fig. 10 LDH release from *Salmonella*-infected bMECs



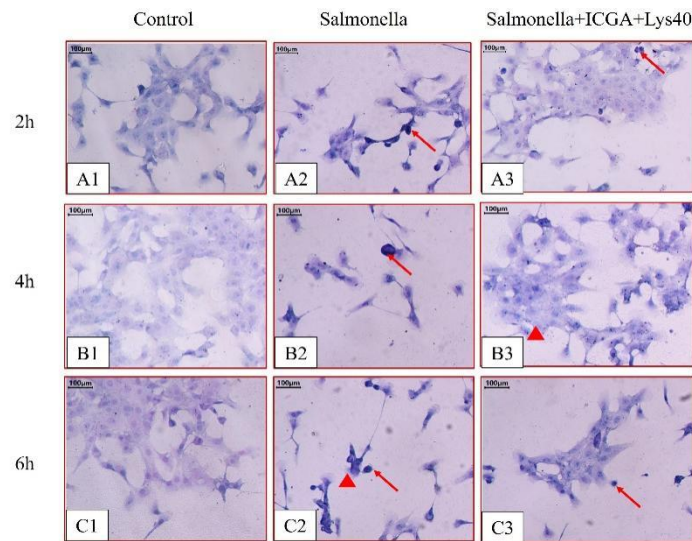
Note: Compared with the Control group: * $P < 0.05$; ** $P < 0.01$; Compared with the Salmonella group: # $P < 0.05$; ## $P < 0.01$

3.3.3 Effects of combination therapy on the morphology and structure of bMECs damaged by *Salmonella*

As shown in Fig. 11, compared with the control group, bMECs presented varying degrees of changes in cellular activity when infected with *Salmonella* at 2 h, 4 h, and 6 h. With prolonged *Salmonella* infection time, the number of bMECs gradually decreased, and bMECs exhibited swelling and nuclear staining, indicating cell damage (Fig. 11 A1, B1, C1, A2, B2, and C2). Compared with those in the *Salmonella* group, the number of bMECs in the *Salmonella*+ICGA+Lys40 group was significantly greater (Fig. 11 A2, B2, C2, A3, B3, and C3). The degree of cell damage in the *Salmonella*+ICGA+Lys40 group was lower than that in the *Salmonella* group. The above experimental results indicate that after *Salmonella* infection of bMECs, inoculation with ICGA and a phage lytic enzyme Lys40 suspension can effectively reduce the damage caused by *Salmonella* to bMECs.

Fig. 11 Morphological structure observation of *Salmonella*-induced bMECs induced by

combination drugs (200× magnification)

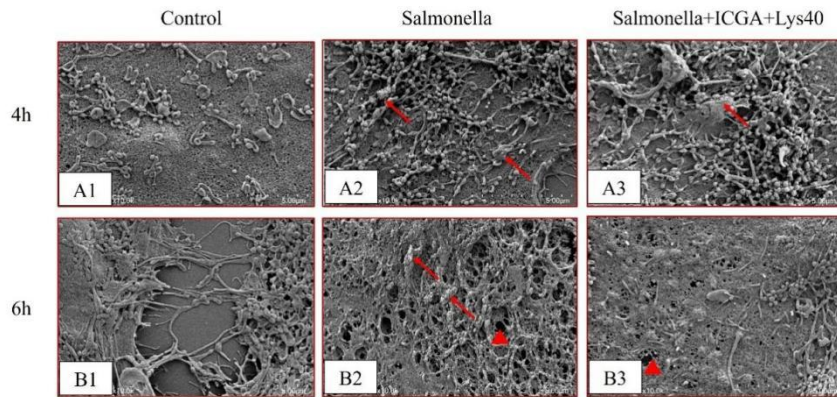


Note: Control group: Control; Salmonella group: Salmonella; Salmonella+ICGA and phage lytic enzyme Lys40 groups: The red arrow in the Salmonella+ICGA+Lys40 group indicates that the nucleus is heavily stained with multinuclei and that the cytoplasm is lightly stained with excessive vacuoles; the red triangle represents cell swelling.

As shown in Fig.12, the structure of bMECs in the control group (Figures A1 and B1) was relatively complete, presenting an elliptical shape with many microvilli growing on the surface and forming many intercellular filaments between cells. Compared with the 4-hour Salmonella group (Figure A2), the 6-hour Salmonella group presented increased adhesion of Salmonella on the membrane surface of bMECs, a collapse of the cell membrane surface, and more significant pathological damage to the number of intercellular filaments and microvilli (Figure B2). Compared with the Salmonella group, the Salmonella+ICGA+Lys40 group (Figure A3) still presented multiple types of damage to the surface of bMECs after 4 hours, and the amount of Salmonella adhesion was significantly lower. In the 6-hour Salmonella+ICGA+Lys40 group (Figure B3), the number of Salmonella adherent bacteria on the cell surface was significantly reduced, and the surface damage to bMECs was lower than that in the Salmonella group.

Fig.12 Microstructural observation of bMECs in combination with drug-induced alleviation of

damage

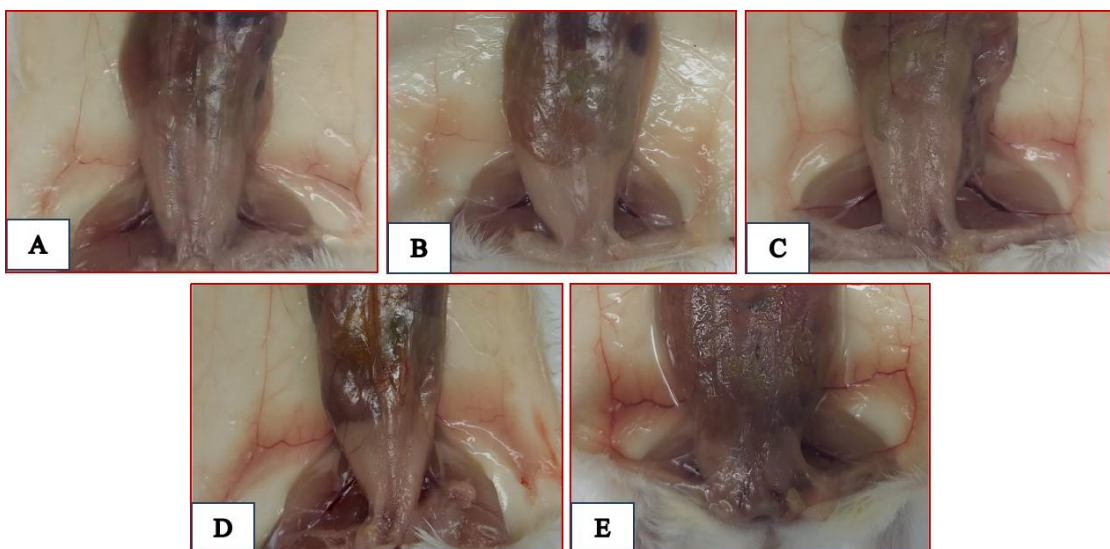


Note: Control group: control; Salmonella group: Salmonella; Salmonella+ICGA and phage lytic enzyme Lys40 groups: the red arrow in the Salmonella+ICGA+Lys40 group represents Salmonella; the red triangle represents the site of epithelial cell damage in the mammary glands of cows.

3.4. Morphological observation of mammary gland tissue in mice infected with *Salmonella*

As shown in Fig. 13, after the inoculation of 100 μ L of different concentrations of *Salmonella* suspension into mouse mammary glands for 24 h, the mammary gland tissues of the control group and the PBS group were milky white in color (Fig. 13A and B). With increasing *Salmonella* suspension inoculation concentration, the color of mouse mammary gland tissues gradually changed from light red to dark red and gray red (Fig. 13C, D, E), indicating that their mammary gland tissues were experiencing inflammation and that the condition was worsening.

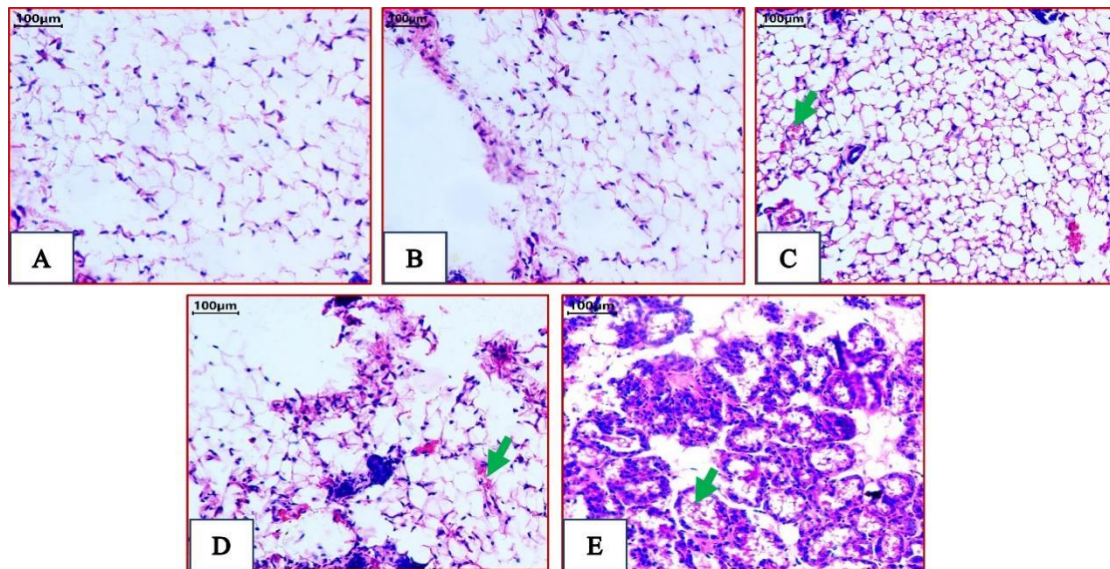
Fig. 13 Gross anatomical morphology of *Salmonella* infection in mouse mammary tissue



Note: A: Control group; B: PBS group; C: 1.5×10^3 CFU/mL; D: 1.5×10^5 CFU/mL; E: 1.5×10^7 CFU/mL

As shown in Fig. 14, Pathological tissue section observation revealed that the mammary gland tissue of the blank control group mice had an intact mammary gland acinar structure, whereas the morphological structure of the mammary gland tissue of the PBS group mice was not significantly different from that of the blank control group (Fig. 14A and B). After infection with different concentrations of *Salmonella*, the structural damage to mouse mammary gland tissue gradually worsened (Fig. 14C, D, and E), manifested as necrosis and shedding of bMECs and the appearance of many cell fragments. With increasing *Salmonella* suspension concentration, the degree of breast connective tissue hyperplasia and fibrosis increased (Fig. 14D and E). On the basis of the comprehensive analysis of mouse mammary gland morphology and HE staining results, a *Salmonella* suspension concentration of 1.5×10^5 CFU/mL was selected for the successful construction of a mouse mastitis model.

Fig. 14 HE staining results of *Salmonella* infection in mouse mammary tissue (H.E. $\times 200$)



Note: A: Control group; B: PBS group; C: 1.5×10^3 CFU/mL; D: 1.5×10^5 CFU/mL; E: 1.5×10^7 CFU/mL. The green arrow in the figure represents the epithelial cells shed from mouse mammary gland tissue.

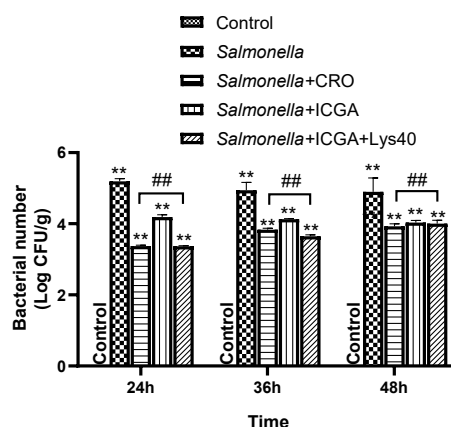
3.5. Therapeutic effect on a mouse mastitis model

3.5.1 Effects of combination therapy on the bacterial load in the breast tissue of mice infected with *Salmonella*

As shown in Fig. 15, after cultivation, the bacterial load in the breast tissue of the control group mice was 0, and there was no growth of *Salmonella* in the plate culture medium

inoculated with the breast tissue grinding solution. The *Salmonella* group presented a greater bacterial load in the mammary gland tissue of the mice, which first increased but then decreased over time. The bacterial loads at 24, 36, and 48 h were approximately 105.5, 105, and 104.5 CFU/g, respectively. Compared with the *Salmonella* group, the *Salmonella*+CRO group presented a significantly lower bacterial load in the mammary gland tissue of the mice ($P<0.01$). The bacterial load in the breast tissue of the mice in the *Salmonella*+ICGA group and *Salmonella*+ICGA+Lys40 group was significantly greater than that in the control group ($P<0.01$). At 24, 36, and 48 hours, the bacterial loads of the *Salmonella*+ICGA group were approximately 103.5, 103.8, and 104 CFU/g, respectively. The bacterial loads of the *Salmonella*+ICGA+Lys40 group were approximately 103.6 CFU/g, 103.9 CFU/g, and 104.1 CFU/g, respectively, and both groups had significantly lower bacterial loads than did the *Salmonella* group ($P<0.01$).

Fig. 15 Effects of ICGA combined with the phage lysase Lys40 on the amount of bacterial carrier in the mammary tissue of *Salmonella*-infected mice



Note: Compared with the control group, the shoulder marker * * indicates $P<0.01$; compared with the *Salmonella* group, the shoulder marker # # indicates $P<0.01$.

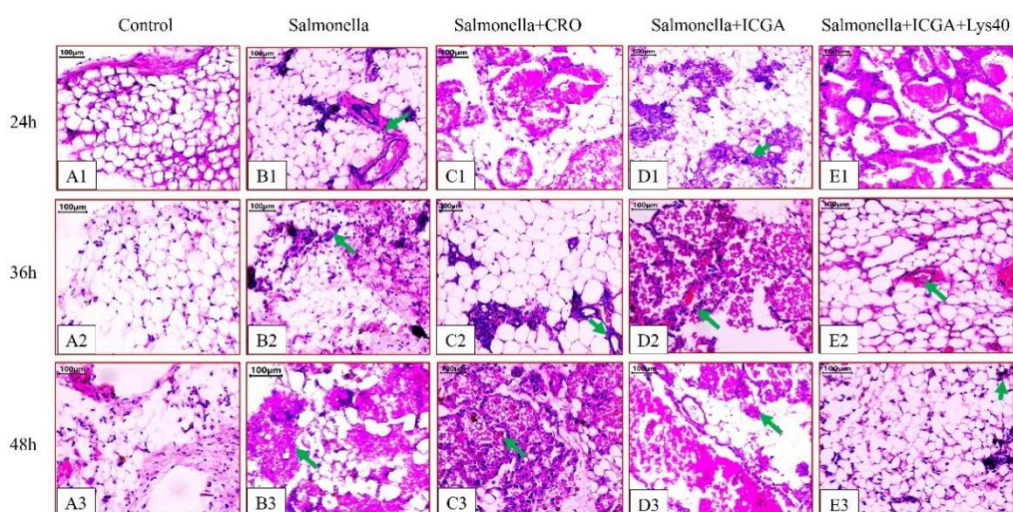
3.5.2 Effects of combination therapy on the morphology of breast tissue in mice infected with *Salmonella*

As shown in Fig. 16, 24 h after inoculation with the *Salmonella* suspension, there was no significant change in the morphology of the breast tissue in Group A (A1, A2, A3). Compared with those in group A, the mammary duct walls of the mice in group B (B1, B2, B3) gradually thickened, and the epithelial cells of the mammary ducts began to shed. When inoculated with *Salmonella* for 24 h, a small number of shed epithelial cells appeared in the mammary ducts of the mice, and the damage was relatively mild (B1). When inoculated with *Salmonella* for 36 and 48 hours, the BMECs of the mice in group B (B2, B3) shed, and the mammary duct wall thickens.

Compared with those in group A, the morphology of mouse mammary gland tissue in groups C and D did not significantly differ 24 h after inoculation with *Salmonella* suspensions (C1, E1). However, at 36 hours and 48 hours after inoculation with the *Salmonella* suspension, a small amount of epithelial cell shedding was observed in the mammary ducts of group C mice (C2, C3). Compared with those in group A, 24 hours after inoculation with *Salmonella* solution, a small number of shed epithelial cells appeared in the mammary ducts of the mice in group D (D1). With prolonged *Salmonella* infection time, the mammary duct wall of the mice in group D gradually thickened, and the number of shed epithelial cells in the mammary ducts gradually increased (D2, D3). Compared with those of group A mice, the mammary duct walls of group E mice slightly thickened, and a small number of epithelial cells (E2, E3) shed from the mammary ducts 36 h after inoculation with *Salmonella* solution.

At each corresponding time point, compared with those in group B, the number of epithelial cells shed from the mammary ducts in the breast tissues of the mice in groups C, D, and E was lower, and the degree of tissue damage was lower than that in group B. Compared with those in groups C and D, at the same time points, the number of epithelial cells shed from the mammary ducts and the degree of breast tissue damage in group E mice were lower than those in groups D and C (E1, E2, E3), indicating that the combination therapy can alleviate breast tissue damage in mice and reduce the shedding of mammary epithelial cells from the breast tissue.

Fig. 16 Effects of ICGA combined with the phage lysase Lys40 on the mammary tissue morphology of *Salmonella*-infected mice (H.E. $\times 200$)



Note: The green arrow in the figure represents the epithelial cells shed from mouse mammary gland tissue.

3.6 Effects of combination therapy on the morphology of breast tissue in mice infected

with Salmonella

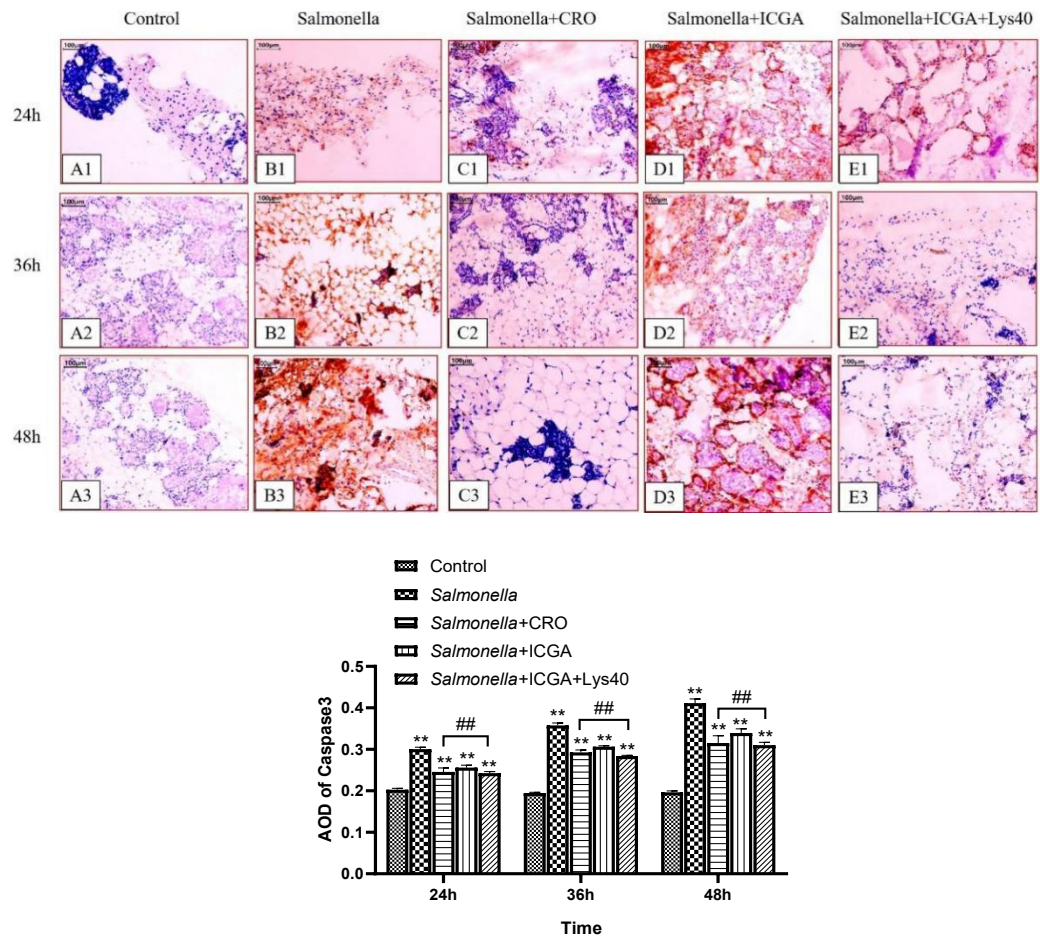
The protein expression level of Caspase-3 in mouse mammary gland tissue is shown in Fig. 17. Compared with those in group A, the AOD values of Caspase-3 expression in the breast tissue of the mice in groups B, C, D, and E significantly increased with increasing infection time ($P<0.01$). Compared with those in group B, the AOD values of caspase-3 protein expression in the breast tissue of the mice in groups C, D, and E were significantly lower ($P<0.01$) with increasing infection time. In summary, Salmonella infection can significantly induce apoptosis in mouse breast tissue cells, and apoptosis activity (referring to the degree of activity involved in initiating programmed cell death) increases with prolonged infection time. C. Treatment with groups D and E can significantly inhibit Salmonella-induced apoptosis of breast tissue cells and reduce their apoptotic activity.

The protein expression level of Caspase-9 in mouse mammary gland tissue is shown in Fig. 18. Compared with those in group A, the expression levels of the Caspase9 protein in mouse breast tissue in groups C, D, and E were significantly greater or significantly greater than those in group A ($P<0.01$ or $P<0.05$). Compared with that in group B, the AOD protein expression of Caspase-9 in the breast tissue of the mice in groups C, D, and E was significantly lower or significantly lower than that in group B ($P<0.01$ or $P<0.05$). These findings indicate that groups C, D, and E inhibit the initiation of apoptosis upstream of the apoptotic pathway by reducing the expression of the apoptosis-initiating molecule Caspase-9, thereby reducing the damage caused by Salmonella infection to breast tissue.

The protein expression level of Cyt-c in mouse mammary gland tissue is shown in Fig. 19. Compared with those in group A, the protein expression levels of Cyt-c in mouse mammary gland tissues in groups C, D, and E were significantly greater or significantly greater than those in group A ($P<0.01$ or $P<0.05$). Compared with that in group B, the expression level of the AOD protein in the Cyt-c of the breast tissue in groups C, D, and E was significantly lower than that in group B ($P<0.01$). These findings indicate that although the intervention treatment of groups C, D, and E did not completely reverse the basic activation of the apoptotic pathway by Salmonella, it significantly inhibited the excessive release of Cyt-c and blocked the increasing trend of apoptosis from the source of initiating apoptosis

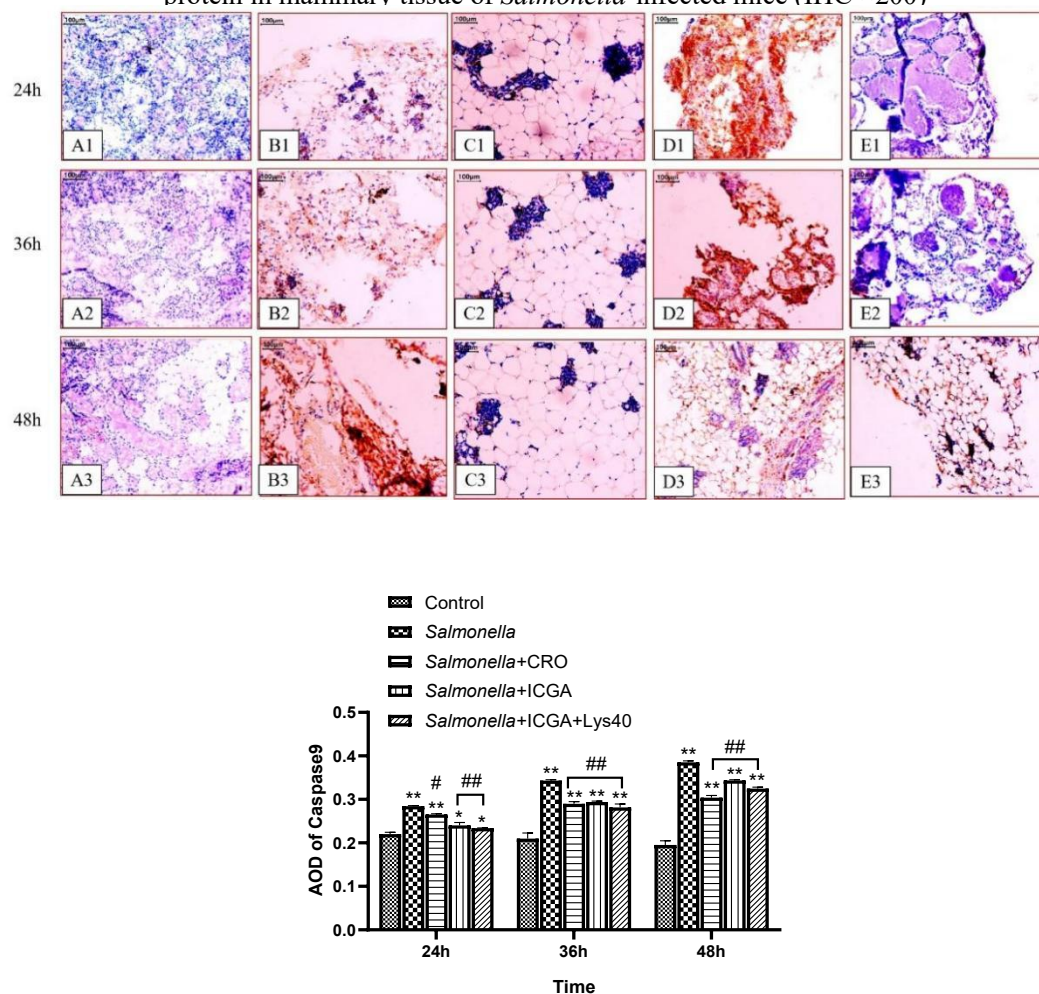
Fig. 17 Effects of ICGA combined with phage lyase Lys40 on the expression of

Caspase-3 protein in mammary tissue of *Salmonella*-infected mice (IHC ×200)



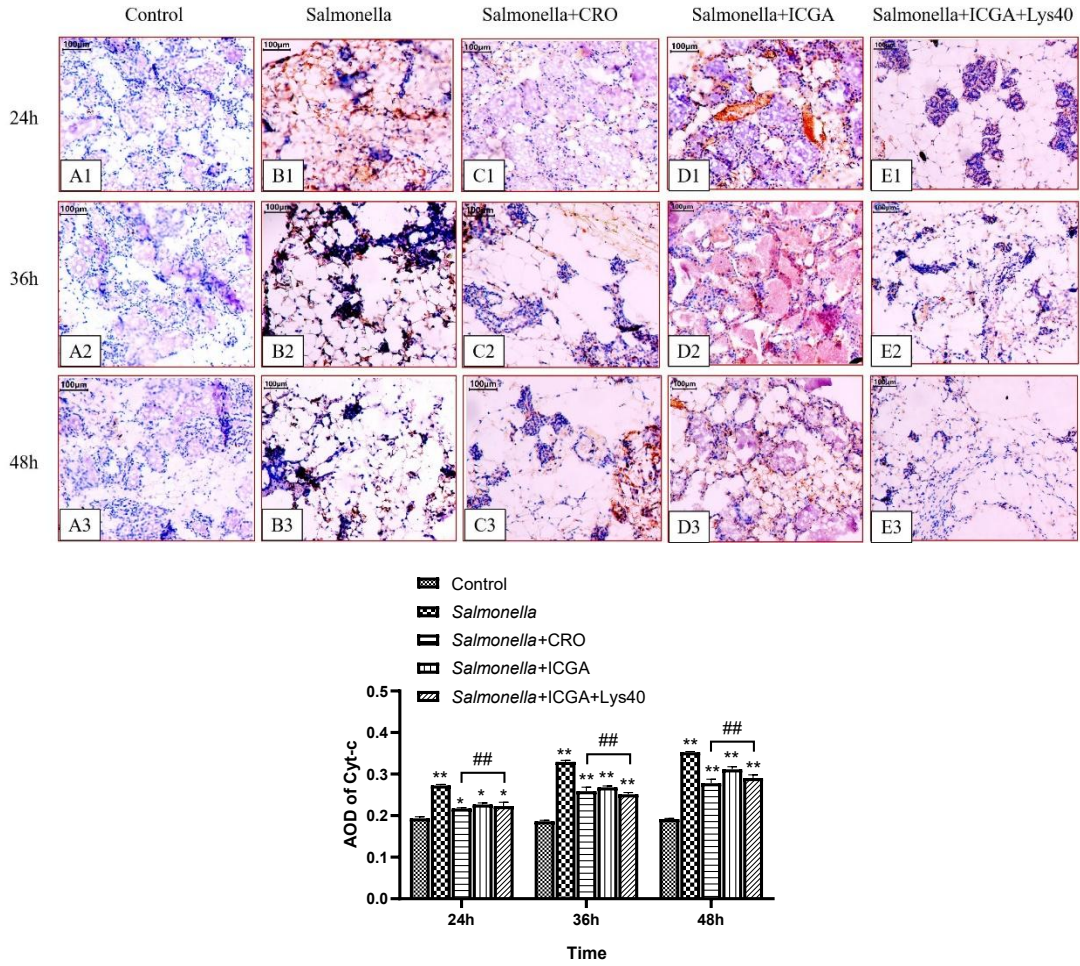
Note: Compared with the Control group, the shoulder marker * * indicates P<0.01; Compared with the Salmonella group, shoulder markers # # indicate P<0.01

Fig. 1 Effects of ICGA combined with phage lyase Lys40 on the expression of Caspase-9 protein in mammary tissue of *Salmonella*-infected mice (IHC $\times 200$)



Note: Compared with the Control group, shoulder markers * * indicate $P<0.01$ and shoulder markers * indicate $P<0.05$; Compared with the Salmonella group, shoulder marker # # indicates $P<0.01$, and shoulder marker # indicates $P<0.05$

Fig. 2 Effects of ICGA combined with phage lyase Lys40 on the expression of Cyt-c protein in mammary tissue of *Salmonella*-infected mice (IHC ×200)



Note: Compared with the Control group, shoulder markers * * indicate $P < 0.01$ and shoulder markers * indicate $P < 0.05$; Compared with the Salmonella group, shoulder markers # # indicate $P < 0.01$

4. Discussion

The growing crisis of antimicrobial resistance (AMR) in veterinary medicine necessitates the urgent development of novel therapeutic strategies that circumvent traditional antibiotics^[9]. This study provides compelling evidence that the combination of a natural plant polyphenol, isochlorogenic acid (ICGA), with the recombinant phage-derived lysin Lys40 constitutes a potent and synergistic antibacterial strategy against *Salmonella*-induced mastitis^[10]. Our integrated *in vitro* and *in vivo* findings not only confirm the efficacy of this combination but also shed light on its multifaceted mechanism of action, which extends beyond direct bactericidal effects to include protection of host tissues^[11].

The profound *in vitro* synergy (FICI = 0.45) against *Salmonella* CVCC-529 underscores the complementary roles of the two agents. While Lys40 alone exhibited limited activity—a common limitation for exogenous lysins against gram-negative bacteria due to the impermeable outer membrane (OM)—ICGA effectively acted as a permeabilizing adjuvant. We propose that ICGA, likely through interactions with lipopolysaccharides (LPS) and the chelation of divalent cations (e.g., Mg^{2+}) that stabilize the OM, compromises its integrity^[12,13,14]. This disruption facilitates the transit of Lys40 to its peptidoglycan substrate, enabling rapid and effective hydrolysis. This "permeabilizer-enzyme" strategy elegantly overcomes a major hurdle in lysin therapy and explains the observed broad-spectrum activity against a diverse panel of mastitis pathogens, including multidrug-resistant strains such as MRSA^[15]. The optimal activity at alkaline pH (8–9) is particularly noteworthy, as it aligns with the elevated pH microenvironment characteristic of mastitic milk, enhancing the translational relevance of this combination for udder therapy^[16].

In addition to direct bactericidal activity, our *in vitro* models using bovine mammary epithelial cells (bMECs) revealed significant cytoprotective effects. The ICGA-Lys40 combination markedly reduced bacterial adhesion and invasion, key initial steps in infection pathogenesis^[17]. This was coupled with a significant reduction in lactate dehydrogenase (LDH) release and preservation of the cellular morphology, indicating the mitigation of bacterium-induced membrane damage and cytotoxicity^[18]. These findings suggest that the combination not only kills bacteria but also actively shields host cells from the destructive cascades in

initiated by infection^[19].

The therapeutic potential of this combination was robustly validated in a murine mastitis model. ICGA-Lys40 treatment significantly reduced the bacterial load in mammary tissue, which was comparable to the efficacy of the broad-spectrum antibiotic ceftriaxone. More importantly, histopathological analysis demonstrated a pronounced alleviation of tissue damage, including reduced epithelial cell shedding and diminished inflammatory infiltration^[19]. A particularly insightful mechanistic discovery from the *in vivo* study was the modulation of the mitochondrial apoptotic pathway^[20,21]. *Salmonella* infection strongly upregulated the expression of key proapoptotic markers (caspase-3, caspase-9, and Cyt-c) in mammary tissue. The ICGA-Lys40 combination significantly downregulated this expression profile. This finding indicates that by achieving rapid and effective bacterial clearance, the treatment prevents the sustained activation of host apoptosis, thereby preserving tissue architecture and function—a critical outcome for maintaining milk-producing capacity in dairy cows^[22,23].

This ICGA-Lys40 strategy addresses several pressing concerns in livestock therapy. First, it offers a "green" antibiotic alternative, leveraging a natural compound and a precisely targeted enzymatic agent, which may reduce the risks of drug residues and environmental contamination associated with conventional antibiotics^[24,25]. Second, the low propensity for resistance development against lysins and the multitarget action of the combination (membrane disruption plus cell wall lysis) present a high barrier for bacterial evasion. Third, the observed antiapoptotic effect highlights an added therapeutic dimension that focuses on host–pathogen interactions and tissue homeostasis, moving beyond mere bactericidal endpoints^[26,27,28].

This study has limitations that point to important future directions. While the permeabilizing role of ICGA is strongly inferred, detailed biophysical studies (e.g., membrane fluidity assays and OM vesiculation analysis) are needed to elucidate the exact molecular interactions involved. The pharmacokinetics, optimal formulation (e.g., stability in milk, udder retention time), and potential *in vivo* toxicity of the combination require thorough investigation in target species (i.e., dairy cows) before clinical application^[29,30,31]. Furthermore, exploring the efficacy against biofilm-embedded bacteria, a common state in chronic mastitis, would be a valuable extension of this work^[32].

The synergistic combination of ICGA and the phage lysin Lys40 represents a highly promising, multimechanistic therapeutic strategy against bovine mastiti

s. It effectively overcomes the gram-negative barrier, exhibits broad-spectrum activity, reduces bacterial pathogenesis at the cellular level, clears infection *in vivo*, and protects mammary tissue by inhibiting infection-triggered apoptosis^[33]. This study lays a solid foundation for the development of a novel, effective, and sustainable enzymatic-based therapy to combat AMR in animal husbandry.

5. Conclusion

This study revealed that ICGA and Lys40 act synergistically to inhibit *Salmonella* and other major mastitis pathogens. This combination effectively reduces bacterial colonization and tissue damage both *in vitro* and *in vivo* while mitigating host apoptosis. This antibiotic-free strategy represents a promising candidate for the treatment of bovine mastitis, addressing the critical issues of AMR and milk safety.

Declarations

Competing interests

The authors declare that they have no competing interests.

Data availability statement

The original contributions presented in the study are included in the article, and further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Institutional Animal Care and Use Committee and the Animal Research Ethics Committee of Hebei Engineering University of China; the research scheme is BER-YXY-2024014.

Author contributions

Shi Yuxiang: Conceptualization and writing—review and editing. Kang Jinzhe and Gao Jiaxin: Writing—original draft and Data curation. Yang Jiawen and Zhang Jing: Formal analysis. Tian Chunyu and Wang Fangfang: Investigation. Liu Jianan and Li Pei: Project administration, Zhong Cuihong and Zhang Xiuying: supervision. All the authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

Consent to Publish declaration:not applicable

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