Supporting Information for

Metal-phenolic self-assembly shielded probiotics in hydrogel reinforced wound healing with antibiotic treatment

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Materials and methods

Materials

Hyaluronic acid (HA, Mn = 960 kDa) was purchased from Bloomage Freda Biopharm Corporation (Shandong, China). Carboxylated chitosan (CCS, degree of carboxylation ≥ 80%) was purchased from yuanye Bio-Technology Corporation (Shanghai, China). Sodium periodate ($NaIO_4$) was purchased from Macklin (Shanghai, China). Ferric chloride ($FeCl_3$) and tannic acid (TA) were obtained from Aladdin Bio-Chem Technology Corporation (Shanghai, China). *Lactobacillus reuteri* (*L. reuteri*) was obtained from China General Microbiological Culture Collection Center (CGMCC). DeMan-Rogosa-Sharpe (MRS) medium, Tryptone Soya Broth (TSB) medium and Live/Dead BacLight Bacterial Viability kits were purchased from Solarbio Science & Technology Corporation (Beijing, China). Dulbecco's Modified Eagle Medium (DMEM) and Fetal Bovine Serum (FBS) were purchased from Thermo Scientific (USA). All mice were procured from the Model Animal Research Center of Nanjing University (Nanjing, China).

Synthesis of *L. reuteri*@FeTA

The *L. reuteri*@FeTA was successfully prepared based on the packaging of metal-phenolic coordination. Briefly, 10^9 CFU *L. reuteri* were washed three times with PBS and suspended in 900 μL PBS (pH 6.0). Then, adding 50 μL $FeCl_3$ (1.25 mg/mL) and 50 μL tannic acid (TA) solution (5 mg/mL) required the vortexing between each addition, respectively. In this study, the DeMan-Rogosa-Sharpe (MRS) medium was used for the culture of *L. reuteri* at 37 ℃ in anaerobic conditions.

Synthesis of hydrogels

As the precursor of hydrogel, oxidized hyaluronic acid (OHA) was synthesized at the first step. Briefly, 2.5 g HA was completely dissolved in 100 mL ultrapure water and followed by the addition of 2.5 g $NaIO_4$ with stirring for 12 h in a dark environment. Then, 3.5 mL ethylene glycol was added and stirred for 30 min. The resulting product was purified through dialyzed in ultrapure water for 3 days and freeze-dried. Then, the hydrogel was successfully prepared by Schiff base reaction based on our reported literature. Briefly, the same volume of 2% (wt/vol) CCS solution and 3% OHA solution were completely mixed at physiological conditions and gelatinization. The obtained hydrogel was named Gel.

For Gel/L hydrogel, 10^6 CFU/mL *L. reuteri* was washed with PBS for three times and dissolved in OHA solution ahead. Then, mix with the same volume of CCS solution at 37 °C for gelatinization. The obtained *L. reuteri*-loaded hydrogel was named Gel-L. The Gel/L@FeTA hydrogel was prepared in the same method. The turbidity tube was employed for bacteria count in this study.

Characterization

The morphology and internal structure of hydrogels and bacteria were observed with a scanning electron microscope (SEM, HITACHI S-800, Japan) and a transmission electron microscope (TEM, JEM-2100Plus, Japan). And the element mappings of Fe (mark *L. reuteri*@FeTA) were taken by the X-ray energy dispersive spectroscopy (EDS) on TEM. The pore size of *L. reuteri* was measured by Nano measurer 1.2, and each sample measured probiotics (n=50) in different areas. Each sample had at least three samples.

The equilibrium swelling ratio of hydrogels was measured by water absorption. Briefly, each hydrogel's dry weight (W_1) was measured and fully immersed in 5 ml PBS (pH 7.4) at 37 ℃ until reaching the equilibrated state, and then weighing them immediately (W_2). Each sample had at least three samples. The equilibrium swelling ratio was calculated by the following formula:

$$\text{Equilibrium swelling ratio (\%)} = (W_2\text{-}W_1)/W_1 \times 100\%$$

The *in vitro* degradation ratio of hydrogels was measured through infiltration in physiological conditions. Briefly, the weight of each initial hydrogel (W_0) was measured and then fully immersed in 15 mL PBS (pH 7.4) at 37 ℃ with shaking at 80 rpm. The samples were removed from PBS and weighing them immediately (W_t) at different times. Each sample had at least three samples. The *in vitro* degradation ratio was calculated by the following formula:

$$\textit{In vitro}\text{ degradation ratio (\%)} = (W_0\text{-}W_t)/W_0 \times 100\%$$

The storage modulus (G′) and loss modulus (G″) of hydrogels was determined by a Dynamic Mechanical Analyzer (DMA, TA-Q800, USA) in a strain ranging (from 0.1 ~1000 %) at a frequency of 1 Hz and a frequency from 0.1 to 10 Hz at constant room temperature, respectively. And the compression performance of the hydrogels was also observed with a universal testing machine (CMT2103, METS, USA) at a strain rate of 5 mm/ min. Each sample had at least three samples.

***In vitro* antibiotic shielding assay of hydrogels**

To evaluate the antibiotic shielding capacity of each sample in common antibiotics, 10^6 CFUs *L. reuteri* or1 mL hydrogel was added into 10 mL gentamicin, penicillin, and cephalosporin solution (100 μg/mL, dissolved by MRS medium) to co-cultured at 37 ℃ in anaerobic conditions. Pure MRS was employed for the control. The optical density (OD) of *L. reuteri* in each group was measured by a microplate reader after 12h, 24 h, and 48 h respectively. Further, 10^6 CFUs *L. reuteri or* hydrogels was added into 10 mL PBS or gentamicin solution (100 μg/mL, with 1% MRS medium) respectively, and co-cultured for 24 h. The hydrogel was stained with Live/Dead BacLight Bacterial Viability kits and observed the bacterial activity of *L. reuteri* was by confocal laser scanning microscopy (CLSM, Leica TCS SP2, Leica Microsystems, Germany). In addition, the incubation solution had been diluted 10^5 times and spread evenly on MRS agar plates for overnight-cultured at 37 ℃, and the number of clones (N) on plates had been counted. To evaluate the ability of *L. reuteri* to secrete organic acids, the supernatant of bacterial culture was collected at 0 h, 4 h, 8 h, 12 h, 24 h, 36 h, and 48 h, respectively. The level of lactic acid secretion in the supernatant was evaluated via the Boxbio®lactic acid content assay kit.

$$\text{Bacterial survival ratio (\%)} = (N\text{-}N_{PBS})/N_{PBS} \times 100\%$$

***In vitro* antibacterial activity**

The *in vitro* antibacterial activity of hydrogel was evaluated by the spread plate method. Briefly, *E. coli* and *S. aureus* were cultured on TSA for 12 hours at 37 °C. And then, a single colony was inoculated in 10 mL TSB and grown in a shaker incubator at 120 rpm at 37 °C. The sterilized hydrogel extract was mixed with bacterial suspension (10^4 CFU/mL) absolutely and incubated at 37 °C. The control group was bacterial suspension without being treated with hydrogel. After 24 h, dilution in the predetermined multiple, the culture solution was dilution in 10^5 times and spread evenly on TSA, then incubated for 24 h at 37 °C. Finally, the number of single colonies in each group was recorded.

***In vitro* anti-inflammatory activity**

For the enzyme-linked immunosorbent assay (ELISA) assay, RAW264.7 cells were cultured in a humidified incubator supplied with 5% CO2, and DMEM supplemented with 1% penicillin-streptomycin as the medium without serum. The inflammation model was induced by 100 ng/mL lipopolysaccharide (LPS) for 24 h. Then, the sterilized hydrogel extract was co-cultivated with RAW264.7 cells (1×10^4 cells/ well), as the control group was only treated by the medium. The cell supernatant in each group was collected after 24 h and the levels of inflammatory cytokines (TNF-α, IL1-β, IL-10, and TGF-β) were determined by using Mouse Interleukin ELISA Kits, respectively. The

extract in this test was sourced from co-cultivated between hydrogel and medium for 5 days.

***In vitro* biocompatibility**

L929 cells and HUVEC were cultured in a humidified incubator supplied with 5% CO_2, and DMEM supplemented with 1% penicillin-streptomycin and 5% fetal bovine serum as the complete medium. The sterilized hydrogel extract was co-cultivated with L929 cells (5×10^3 cells/ well), as the control group was only treated by the complete medium.

For the CCK8 assay, the *L. reuteri* (10^6 CFUs/ ml) or hydrogel extract was changed to fresh media with 10% Cell Counting Kit-8 (CCK8) after 1 day, 3 days, and 5 days, respectively. After incubation for 2 h, the optical density (OD) of each group was measured at a wavelength of 450 m. The Cell viability was measured by the following formula:

$$\text{Cell viability (\%)} = OD_H/OD_C \times 100\%$$

where OD_H was for each hydrogel group and OD_C was for the control group.

For FDA/PI staining assay and Rhodamine Phalloidin/DAPI staining assay, the L929 cells in each group were stained and then visualized by a fluorescence microscope after incubation for 5 days. FDA can mark the living cell in green and PI can mark the dead cell in red. Rhodamine Phalloidin can mark the cytoskeleton in red and DAPI can mark the nucleus in blue.

For cell migration assay, 10^5 cells L929 cells, and HUVEC were cultured in serum starvation and reached 80% confluence, using a sterile 200 mL pipette tip to create the cell scratches and washing by PBS for 3 times. Then, incubation with hydrogel extracts for 0 h, 12 h 24 h, respectively. The L929 cells were stained by FDA and visualized by a fluorescence microscope. The wound area was measured by Image J software and the closed rate was calculated by the following formula:

$$\text{Closed rate (\%)} = (A_0 - A_t)/A_0 \times 100\%$$

Where A_0 was the wound area in 0 h and A_t was the wound area at a predetermined time.

***In vivo* wounding healing assessment**

The male BALB/c mice (25 g ± 5 g, 6-8 weeks age) were employed to evaluate the *in vivo* wounding healing assessment of hydrogels in the therapy with antibiotic and non-antibiotic therapy, respectively. **All animal experiments were carried out in accordance with current guidelines for the care of laboratory animals and were approved by a proper committee of Sichuan University (KS2020330).** All mice were randomized into four groups and adapted to the breeding environment for 1 week. Then, the mice were anesthetized through intraperitoneal injections for chloral hydrate (0.5 mg/kg body weight). A full-thickness skin round wound (10 mm) was created on the back of each mouse under aseptic conditions. For the group by no-antibiotic therapy, the wounds were treated with pure *L. reuteri*, Tegaderm™, and each sterilized hydrogel, respectively. For the group therapy with antibiotics, 100 ug/mL gentamicin was added to the surface of the wound and had the same treatment as the normal wound group. The wounds' contraction was measured and imaged at a predetermined time and measured by Image J software. The wound area recovery closure (RC) was measured by the following formula:

$$RC\ (\%) = (R_0 - R_1)/R_0 \times 100\%$$

where R_0 is the wound area created, and R_1 is the wound area at a predetermined time.

Histologic analysis

To evaluate the histologic and immunohistochemistry of wound tissue, mice were euthanized at a predetermined time and the wound tissues were collected in 4% (vol/vol) paraformaldehyde for 24 h. Then dehydrated by graded alcohols and embedded in paraffin. Each paraffin was cross-sectioned into 4 μm thickness slices and stained with the hematoxylin and eosin (H&E), Masson's trichrome, and immunohistochemistry (TNF-α, TGF-β). The collagen deposition and expression levels of TNF-α, and TGF-β were analyzed by Image J software.

Western-blots assays

To evaluate the expression of angiogenesis-related proteins in wound tissue. The skin tissue was crushed in liquid nitrogen and then homogenized in Radio Immunoprecipitation Assay (RIPA) lysis buffer for 30 min. The obtained tissue protein was collected, quantified, electrophoresed, transmembrane, incubated with related antibodies and visualized by Enhanced Chemiluminescence (ECL) imaging system. The expressions of VEGF and CD 31 in skin tissues were analyzed by Image J software and the β-actin as a protein loading control.

Statistical analysis

The results were expressed as the arithmetic mean ± standard deviation. And three samples were drawn for statistical analysis at least. All of the data were performed statistically analyzed in SPSS 22.0 software. When $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.001$ (***), the differences were considered significant, and $p > 0.05$ (#) showed no significant difference.

Results and discussion

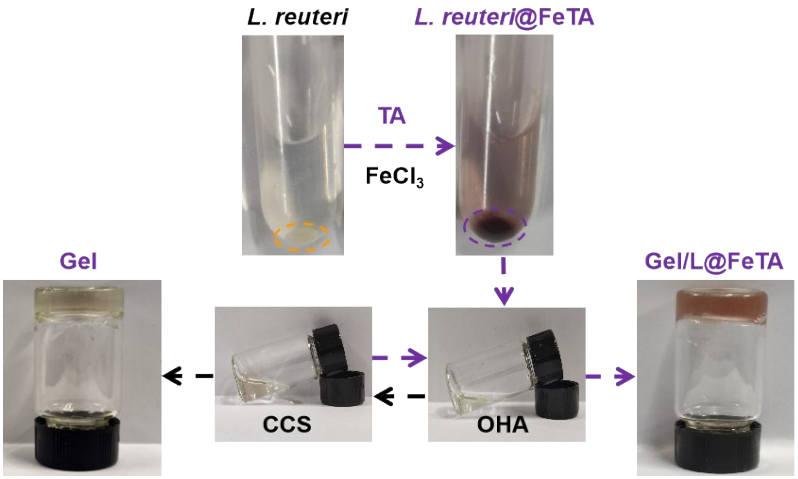

Fig. S1. The intuitive images of the synthesis process for Gel (black arrow) and Gel/L@FeTA (purple arrow).

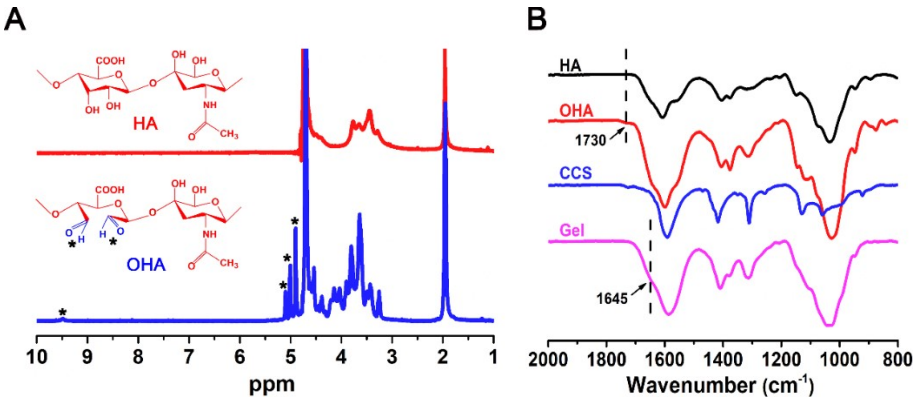

Fig. S2. (A) The ^{1}H NMR spectrum of HA and OHA. (B) The ATR-FTIR spectrum of HA, OHA, CCS, and Gel.

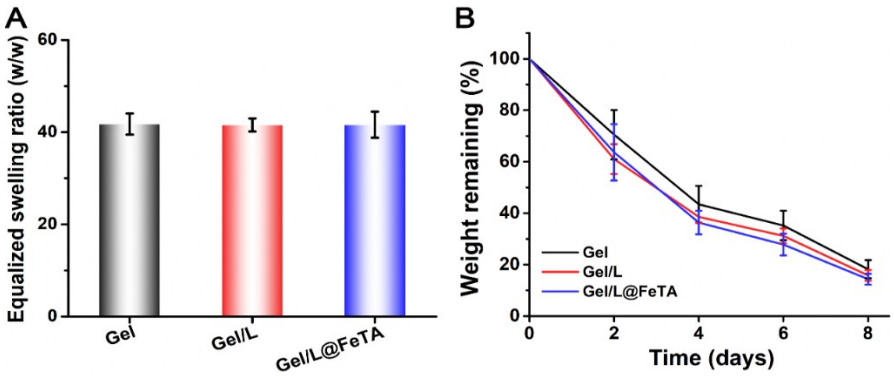

Fig. S3. The equilibrium swelling ratio (A) and the degradation ratio (B) of hydrogels.

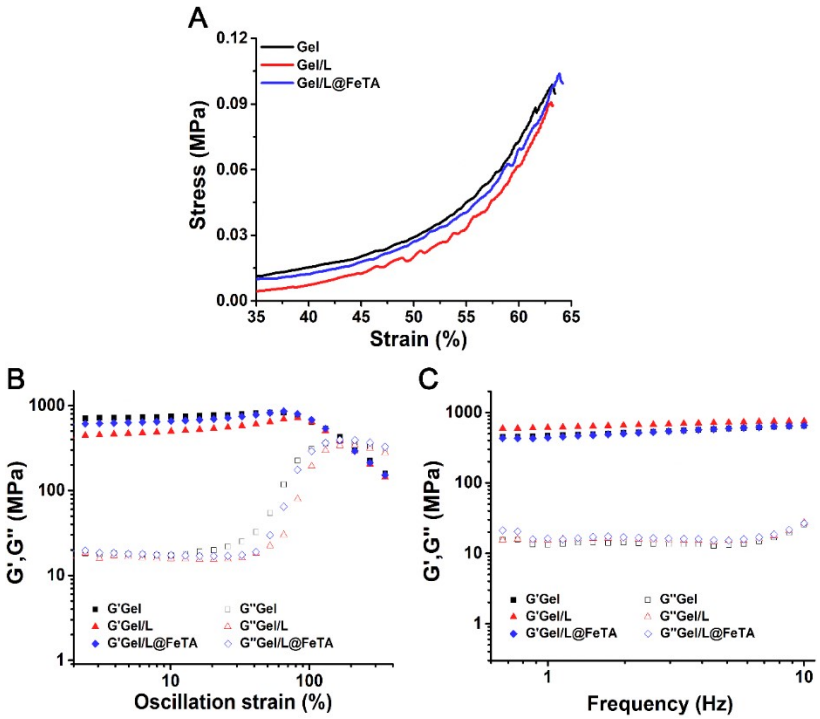

Fig. S4. The compressive stress-strain curves (A), strain sweep test (B), and frequency sweep test (C) of hydrogels.

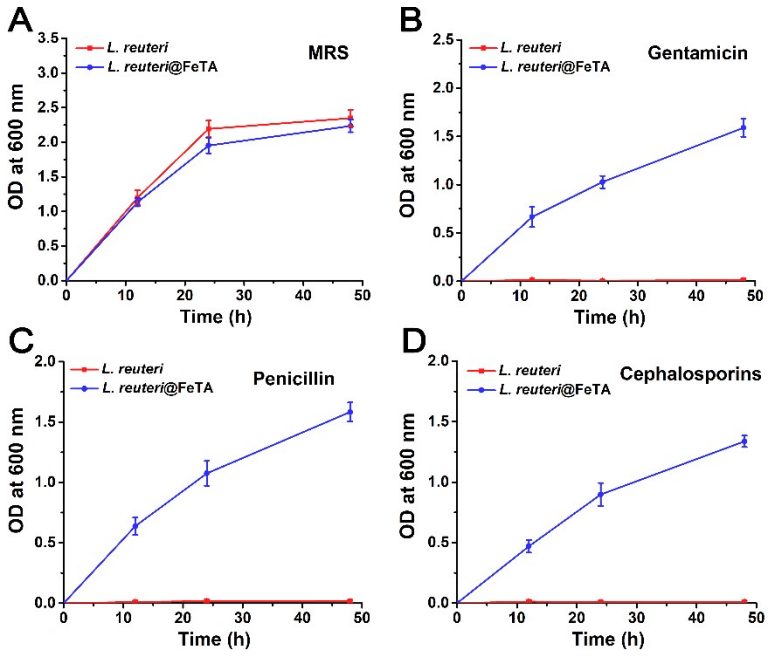

Fig. S5. Growth curve of *L. reuteri* and *L. reuteri*@FeTA under the antibiotic influence: (A) MRS, (B) gentamicin, (C) penicillin, and (D) cephalosporins.

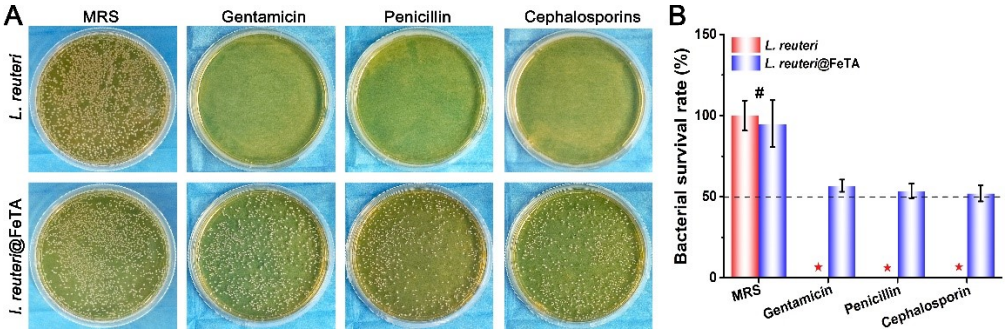

Fig. S6. Representative images (A) and bacterial survival ratio (B) of the *L. reuteri* growth under sample influence. ★= 0, $p > 0.05$ (#).

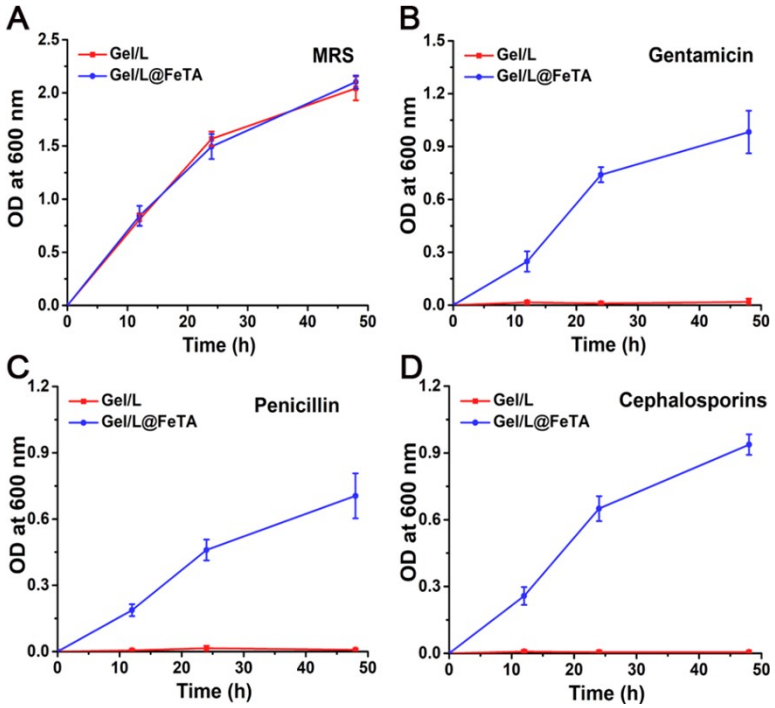

Fig. S7. Growth curve of *L. reuteri* in hydrogel under the antibiotic influence: (A) MRS, (B) gentamicin, (C) penicillin, and (D) cephalosporins.

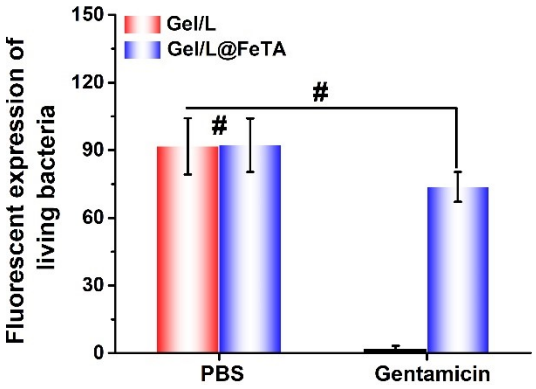

Fig. S8. The Fluorescent expression of living bacteria under sample influence.

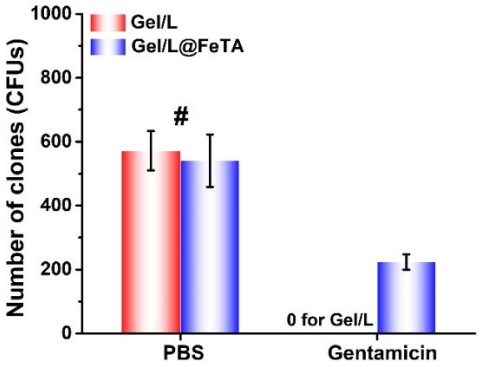

Fig. S9. The statistics of clones under sample influence.

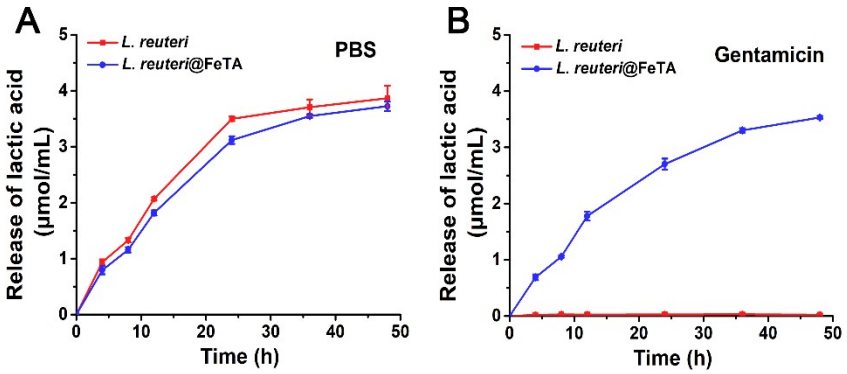

Fig. S10. The release of lactic acid from *L. reuteri* and *L. reuteri*@FeTA in PBS (A) and gentamicin (B).

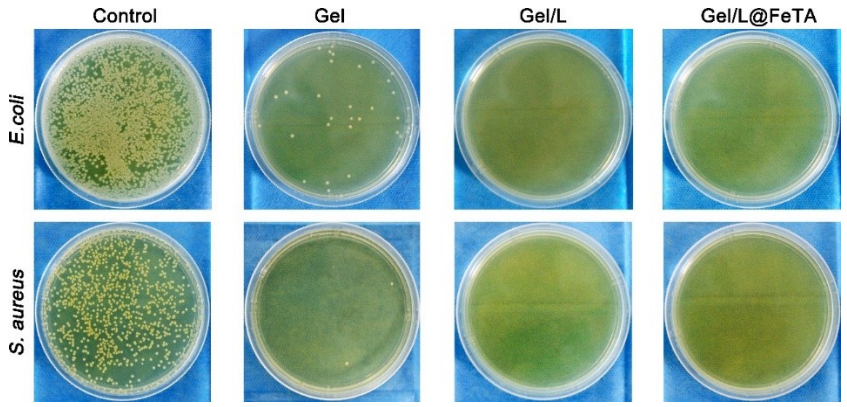

Fig. S11. Images of bacteria clones on agar plates after contact with hydrogel extract.

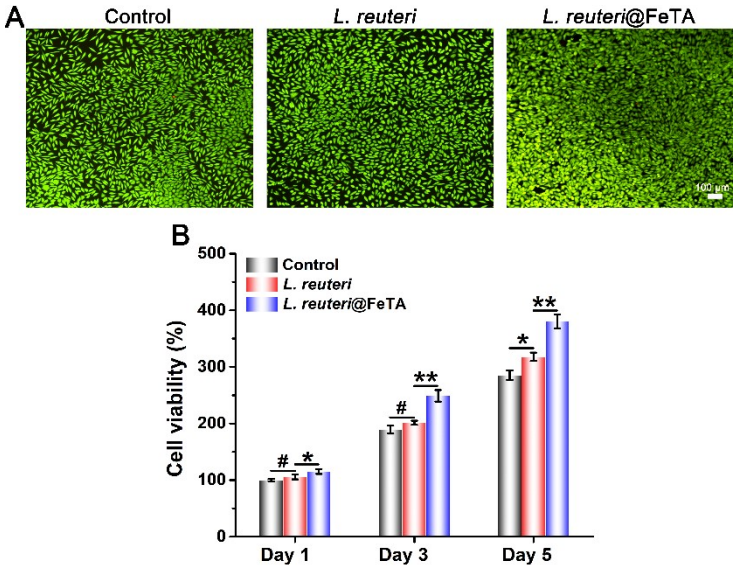

Fig. S12. (A) The microscopic images of Live/Dead stains for L929 cells after 5 days incubated by *L. reuteri* and *L. reuteri*@FeTA extract. (B) The viability of L929 cells incubated by *L. reuteri* and *L. reuteri*@FeTA extract. $p < 0.05$ (*), $p < 0.01$ (**), or $p > 0.05$ (#), n=3.

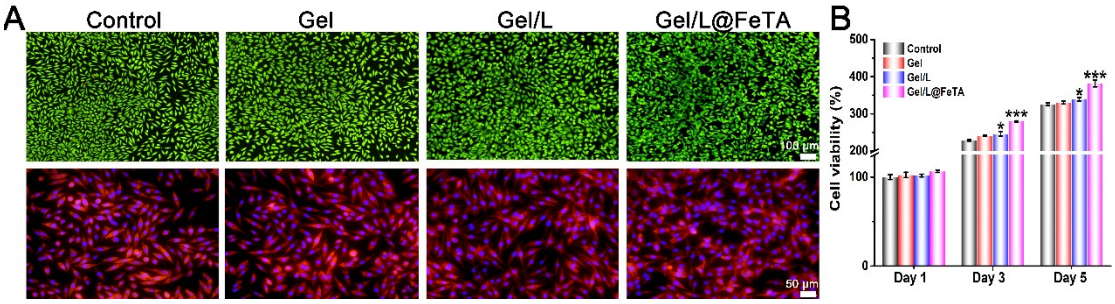

Fig. S13. (A) The microscopic images of Live/Dead stains and cytoskeleton & nuclear stains of L929 cells after 5 days incubated by hydrogel extract. (B) The viability of L929 cells incubated by hydrogel extract. $p < 0.05$ (*), or $p < 0.001$ (***), n=3.

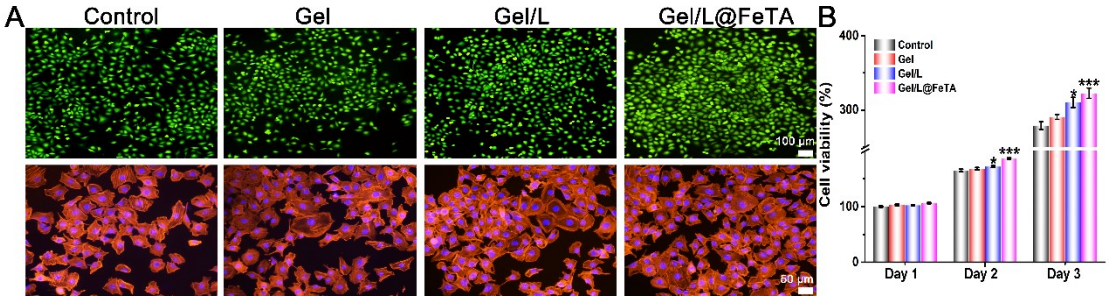

Fig. S14. (A) The microscopic images of Live/Dead stains and cytoskeleton & nuclear stains of HUVEC after 3 days incubated by hydrogel extract. (B) The viability of HUVEC incubated by hydrogel extract. $p < 0.05$ (*), or $p < 0.001$ (***), n=3.

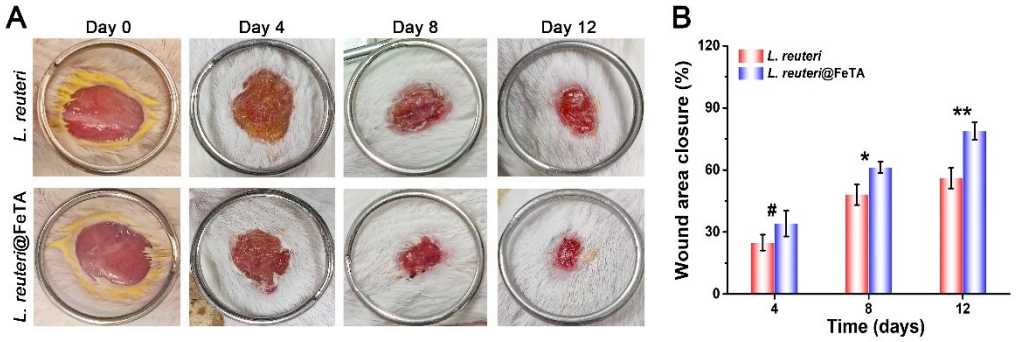

Fig. S15. Representative image (A) and statistics (B) of wounds healed at different times by treated with *L. reuteri* and *L. reuteri* @FeTA. The above results were derived from the therapy with gentamicin. $p < 0.05$ (*), $p < 0.01$ (**), or $p > 0.05$ (#), n=3.

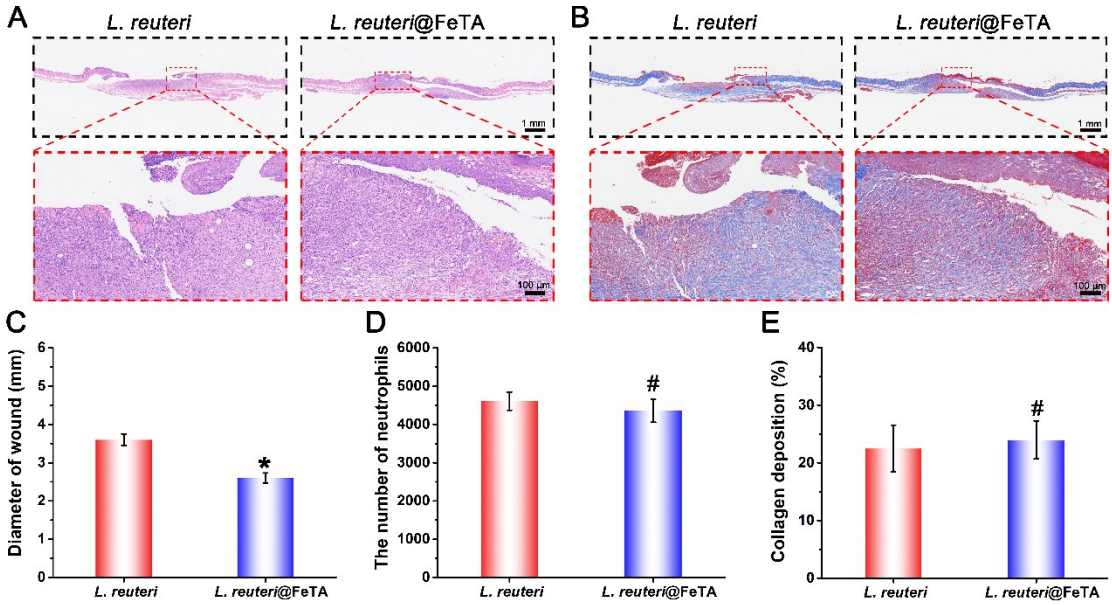

Fig. S16. Images of H&E stained (A) and Masson's trichrome stained (B) wound tissue at 12 days respectively. (C) The wound diameter statistics of tissue at 12 days. (D) The neutrophils' statistics of wound tissue at 12 days. (E) The collagen deposition rate of wound tissue at 12 days. The above results were derived from the therapy with gentamicin. $p < 0.05$ (*), or $p > 0.05$ (#), n=3.

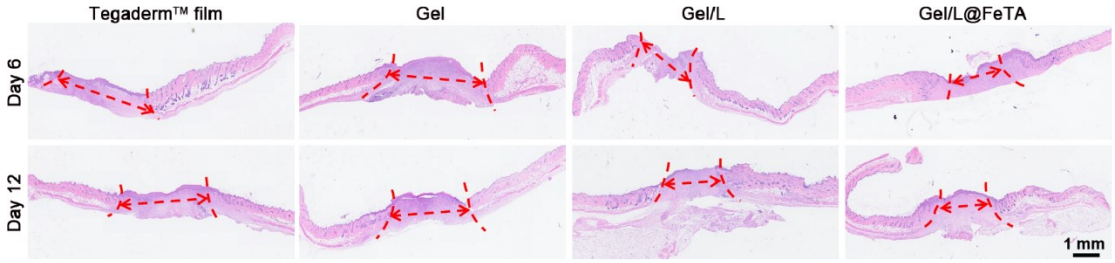

Fig. S17. The location of wound area in HE straining section by therapy of antibiotic.

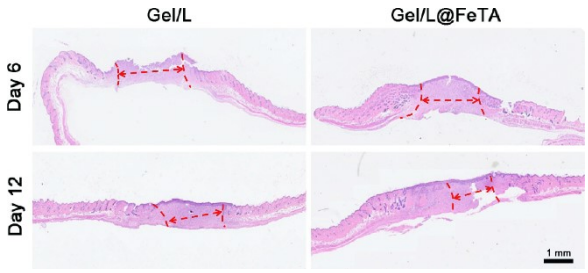

Fig. S18. The location of wound area in the HE straining section by no-antibiotic therapy.

Tab. S1. The wound diameter statistics of tissue in the therapy of antibiotic

	Wound diameter in 6 days (mm)	Wound diameter in 12 days (mm)
Tegaderm™ film	4.9 ± 0.08	3.6 ± 0.11
Gel	3.6 ± 0.11	2.8 ± 0.05
Gel/L	3.2 ± 0.12	2.6 ± 0.12
Gel/L@FeTA	2.3 ± 0.06	1.8 ± 0.06

Tab. S2. The wound diameter statistics of tissue in no-antibiotic therapy

	Wound diameter in 6 days (mm)	Wound diameter in 12 days (mm)
Gel/L	2.2 ± 0.06	1.8 ± 0.13
Gel/L@FeTA	2.1 ± 0.1	1.6 ± 0.10

Tab. S3. The number of new hair follicles of wound area the therapy of antibiotic

	new hair follicles in 6 days	new hair follicles in 12 days
Tegaderm™ film	0.3 ± 0.58	1.0 ± 1.00
Gel	0.7 ± 0.58	1.7 ± 0.58
Gel/L	1.7 ± 1.15	2.3 ± 0.58
Gel/L@FeTA	2.7 ± 2.52	8.7 ± 1.53

Tab. S4. The number of new hair follicles of wound area in no-antibiotic therapy

	new hair follicles in 6 days	new hair follicles in 12 days
Gel/L	2.0 ± 1.00	7.0 ± 1.73
Gel/L@FeTA	2.3 ± 0.58	8.0 ± 1.0