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Limosilactobacillus fermentum D0S accelerates skin wound healing through improving immune microenvironment and promoting collagen synthesis and re-epithelialization

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Abstract

Wound healing is a complex process that occurs in a dynamic and constantly changing microbial environment. Emerging evidence emphasizes the crucial role of site-specific microorganism mediated signal transduction and inflammatory response in shaping wound outcomes. In this study,we isolated an oral probiotics *Limosilactobacillus fermentum* D0S strain and investigated its potential role in promoting skin wound healing and mechanism involved. Antimicrobial assays demonstrated that D0S significantly reduced the abundance of pathogenic bacteria such as *Staphylococcus aureus* and *Staphylococcus epidermidis*, rather than *Pseudomonas aeruginosa*. Biofilm, extracellular polysaccharide (EPS) detection and co-focal microscopy assays showed that D0S markedly reduced the biofilm and EPS formation abilities of the three pathogenic bacteria. Scanning electron microscope examination showed that D0S efficiently changed the three pathogenic bacterial biofilm structure from dense to loose. Through *in vivo* skin wound healing experiment, we found that D0S significantly accelerated wound healing, as evidenced by increased collagen synthesis and re-epithelialization and

reduced inflammatory responses. Mechanism study indicated that D0S could regulate the wound microenvironment through efficiently down-regulating the pro-inflammatory IL-6 and IL-1β expression and up-regulating the anti-inflammatory mediator IL-1RA/IL1-RN expression, thereby promoting wound healing. This study provides a new probiotic *Limosilactobacillus fermentum* D0S strain and its therapeutic potential for skin wound healing.

Keywords: skin wound healing; *Limosilactobacillus fermentum*; probiotics;*immune microenvironment*

Introduction

Skin wound healing is a highly coordinated process involving immune signaling, microbial ecology and tissue regeneration. Traditionally, previous researches focus on the roles of growth factors, scaffolds and re-epithelialization mechanisms. However, emerging studies now emphasize the importance of the immune-microbiome axis in wound healing (He et al., 2026). Interestingly, oral mucosal wound exhibits superior healing rate than skin wound with less inflammatory response, and minimal scar formation (Lee & Eun, 1999).This phenomenon is attributed not only to specific cell types involved in wound repair but also to the significant contribution of certain resident microbial communities on the mucosal surface (Mak et al., 2009). The oral microbiota plays a critical role in promoting tissue immunity(Verma et al., 2018), repairing mechanisms and defending against pathogens(Han et al., 2020). Therefore, we hypothesize that probiotics in the oral cavity may play an important role in promoting oral wound healing (Jiang Foong et al., 2026); even they could exert a similar promoting effect on the skin(Sekar et al., 2026).

Previous studies have demonstrated that specific probiotic strains including *Lactobacillus rhamnosus* (*L. rhamnosus*), modulate dermal immune cell density and IL-10 expression levels, effectively counteracting ultraviolet radiation-induced damage(Zhang et al., 2018). Lactobacillus strains has been demonstrated to effectively reduce or eradicate pathogen loads(Ong et al., 2020), decrease necrotic tissue, expedite the formation of granulation tissue, diminish the wound area and facilitate wound healing(Dubey et al., 2021).Those findings substantiate the capacity of probiotics to exert significant immunomodulatory effects within cutaneous tissues(Lukic et al., 2017).

In this study, we isolated an oral probiotic *Limosilactobacillus fermentum* (*L. fermentum*)D0S strain, investigated its effects on skin wound healing and underlying mechanism involved by using *L. rhamnosus* LGG strain as control. We found that D0S significantly accelerated wound

healing, as evidenced by increased collagen synthesis and re-epithelialization and reduced inflammatory responses.

Materials and Methods

Bacterial strains, eukaryotic fibroblastic and skin epithelial cell lines and growth conditions

This study isolated various *Lactobacilli* / *Limosilactobacillus* strains from oral cavity and screened out several promising probiotic strains based on 16S rRNA sequencing analysis for subsequent experiments. Pathogenic bacterial strains such as *Staphylococcus aureus* (*S. aureus*, ATCC 25923), *Staphylococcus epidermidis* (*S. epidermidis*, CICC 10436) and *Pseudomonas aeruginosa* (*P. aeruginosa*, ATCC 10145) and probiotics *L. rhamnosus* LGG (ATCC 53103) strains were grown in Brain Heart Infusion (BHI) broth medium at 37 ℃. *L. rhamnosus* LGG strain was grown in MRS medium at 37 ℃. All bacterial strains were stored at -80 ℃ before use.

For eukaryotic fibroblastic and skin epithelial cell lines, mouse fibroblast line L929 and human immortalized keratinocytes HaCaT were both purchased from the American Type Culture Collection (ATCC), cultured with DMEM containing 10% FBS and 1% penicillin-streptomycin, and stored in liquid nitrogen in our laboratory. All cells in the logarithmic growth phase were used for the following experiments.

Agar diffusion assay

The antibacterial activity of the six isolated oral probiotic strains was tested using the standard strain of *S. aureus, S. epidermidis and P. aeruginosa*. The pathogenic bacterial strains were cultured overnight for this experiment. Then evenly spread on BHI agar plates using sterile cotton swabs. Three wells with a diameter of 7 millimeters were drilled on the plates. Six strains of oral probiotics and the LGG strain were cultured overnight on MRS medium until they reached the logarithmic growth phase. The OD_{600} values were adjusted to 1. The dead bacterial suspension was prepared by boiling at 95 ℃ for 1 h. Then, live oral probiotic strain and dead oral probiotic strains were deposited in 100 μL in the wells, MRS medium served as negative control. After culturing at micro-aerobic conditions at 37 ℃ for 24 h, the inhibition zone (IZ) was reported as the growth inhibition diameter (the well diameter was subtracted).

Growth curve determination

The *L. fermentum* D0S strain was cultured in MRS broth medium under aerobic conditions at 37 ℃ for 18-24 h. *S. aureus, S. epidermidis and P. aeruginosa* were cultured in BHI broth medium under aerobic conditions at 37 ℃ for 18-24 h. The suspension concentrations of each strain were determined and adjusted using spectrophotometry to achieve a final concentration of approximately 10^8 CFU/mL. The bacterial suspensions of each strain were diluted 1:100 in BHI medium and thoroughly mixed by vigorous shaking. In the co-culture system, *L. fermentum* D0S was inoculated in equal volumes (1:1 v/v) with each pathogenic bacterium. A total of 200 μL of reaction mixture was added per well in triplicate in a 96-well plate. The growth dynamics were continuously monitored using a 37 ℃ constant temperature microbiology plate reader with automatic collection of OD_{600} absorbance values every 30 min for 12 h until the microbial community entered the stationary growth phase. Experimental groups were designed as follows: D0S alone group, D0S co-culture with each pathogen group, each pathogen alone group, and sterile medium control group (MRS as blank). Growth curves of the co-culture systems were plotted. Each experiment was independently repeated three times.

Bioflim formation detection by crystal violet staining

The bacterial suspension concentrations of *S. aureus, S. epidermidis, P. aeruginosa* and *L. fermentum* D0S were adjusted to approximately 10^8 CFU/mL using spectrophotometry. Each bacterial suspension was diluted at a ratio of 1:100 in BHI medium and thoroughly mixed by vigorous shaking. A co-culture system was established on BHI medium containing 1% glucose: *L. fermentum* D0S and pathogenic bacteria were inoculated in equal volumes (1:1 v/v). A total of 200 μL of culture medium was added per well in a 96-well plate. Experimental groups included: D0S alone, D0S co-culture with pathogenic bacteria, pathogenic bacteria alone, and sterile medium control group. The assay was performed in technical triplicates. After incubation at 37 ℃ for 24 h, supernatants were discarded, and the plates were gently washed three times with PBS followed by liquid aspiration. Each well was fixed with 200 μL anhydrous ethanol for 15 min, dried and stained with 0.01% crystal violet dye solution for 30 min. After removing the dye solution, the plates were washed three times with PBS, dried and photographed. Biofilm quantification was performed by dissolving crystal violet staining using 120 μL 33% acetic acid solution for 30 min, followed by aspiration of 100 μL elution buffer and transfer to a new 96-well plate. OD_{575} was measured using a microplate reader (BioTek Instruments, Winooski, VT, USA). The inhibition efficiency of biofilm formation was evaluated through three independent experiments.

Extracellular polysaccharide (EPS) quantification by anthrone-sulfuric acid method

As described in Method 2.4 to prepare the co-culture systems containing D0S and pathogenic bacteria in equal volumes (1:1 v/v). 1 mL mixture was added to each well of a 12-well plate, and incubation was performed under aerobic conditions at 37 ℃ for 24 h. At indicated time points, the supernatants were gently removed, and the biofilm was gently washed three times with pre-cooled PBS buffer. Subsequently, one milliliter of PBS was added to each well, and the biofilm was resuspended then collected in 1.5 mL centrifuge tubes. Centrifugation was performed at 10,000 g for 10 min at 4 ℃, followed by supernatant removal. The precipitate was resuspended in 1 mL PBS and vortexed for 30 s. The washing procedure was repeated twice (each cycle involving centrifugation at 1,000 g for 10 min at 4 ℃ followed by supernatant removal). Residual liquid was aspirated as thoroughly as possible. One milliliter of 0.4 mol/L NaOH solution was added to the precipitate, vortexed thoroughly to resuspend it, and incubate in a water bath at 37 ℃ for 2 h to alkalize and hydrolyze the water-insoluble extracellular polysaccharides. After incubation, centrifuge (10,000 g, 10 min, 4 ℃), 200 μL of supernatant were collected and transferred to a new centrifuge tube. 600 μL of freshly prepared anthrone-concentrated sulfuric acid reagent (containing 0.1% anthrone and 80% concentrated sulfuric acid) were slowly added along the tube wall, then gently mixed well and keep the tube in a water bath at 95 ℃ for 6 min. After cooling to room temperature, 100 μL of reaction mixture were transferred to a 96-well plate and measured at OD_{625} using a microplate reader (BioTek).

Scanning electron microscope examination

The bacterial co-culture samples were prepared using the Method 2.4 described previously. After the formation of biofilm, the bacteria co-culture samples adhering to the cell slides were taken for fixation. The samples were fixed with 3% glutaraldehyde at 4℃ overnight. After fixation, the samples were dehydrated using a gradient of 10% to 100% ethanol and then air-dried at the critical point (K850, Quorum Technologies Ltd., London, UK). The dried samples were imaged using a scanning electron microscope (SU800, Hitachi， Tokyo, Japan) at a voltage of 5 kV and 10kV.

Multiplicity of infection (MOI) determination assay

Following the procedure of CCK8 assays to inoculate 5×10^3 L929 cells per well per 100 μL medium or 1×10^4 HaCaT cells per well per 100 μL complete medium in 96-well plates overnight, at the following day, different MOIs (0, 10, 50, 100) of D0S were added into each well, then at the indicated time points, OD_{450} was recorded to determine optimal MOI.

For wound healing assay, 3×10^5 cells were seeded in a well of 6-well plate with 2 mL complete medium. When the cells had grown 100% confluence, the medium was changed to serum-free DMEM medium for 24 h. Then a wound was made by scratch using a 200 μL pipette tip. After removing the floating cells by PBS, and photographed immediately with a microscope recording as "0 h". Subsequently, different MOIs of D0S were added for co-culture. After co-culturing with bacteria for 8 h, the cells was washed three times with PBS to remove the bacteria, after treatment 16 h (24 h after the cell scratch), and the same scratch area was photographed again. Cell migration capacity was quantitatively assessed through wound wound healing rate (%) calculated using the formula: [(initial scratch area - 24 h scratch area) / initial scratch area] × 100%. Taken together of the results of CCK8 and wound healing assays, the optimal MOI value of D0S was selected for the subsequent experiments. The other oral probiotic strains were used the same dose of MOI as D0S.

Wound healing assay

The wound healing assay was conducted using mouse fibroblasts L929 and human immortalized keratinocyte cells HaCaT to evaluate the effects of different oral probiotics treatments on cell migration ability. A total of 7 groups were set up：the negative control group (NC) and oral probiotics treatment groups (D4A, D1C, D0S, D3B, D1D, D17A). The cells were inoculated at a density of 3×10^5 per well in the culture plate and cultured until 100% confluence. Then, following the precedure described above to perform the wound healing assay and calculate the effect of oral probiotcs at selected dose of MOI on fibroblast and epithelial cell migration capacity.

CCK8 assay

Cell proliferation was also measured using the cell counting CCK8 assay. Briefly, L929 cells at a density of 5000 cells per well, or HaCaT cells at density of 10,000 cells per well were seeded into 96-well plates, and with each sample having three replicates. The next day, CCK8 assay was performed to record the initial OD_{450}. Then, MOI=10 for L929 and MOI=50 for HaCaT of D0S were determined to the well plates for stimulation for 8 h, readings were measured at 12 h, 24 h, 36 h and 48 h after stimulation, with DMEM used as a blank control. At each time point, 10 μL CCK8 reagent (Beyotime, Shanghai, China) was added to each well and continued to incubate for 1 h at 37 ℃. The OD_{450} value was recorded by an enzyme-labeled apparatus (BioTek, USA).

qPCR

Total RNA was extracted from cells using TRIzol reagent, and the concentration of RNA was measured with NanoDrop 8000. After reverse transcription, real-time PCR was performed using the SYBR Green master mix (Abclonal, Wuhan, Hubei, China) with primer pairs and 50 ng cDNA as a template. $2^{-\Delta\Delta Ct}$ method was used to analyze the data. GAPDH was used as a reference to normalize the expression level of each target gene.

Table1. qPCR primer sequences used in this study.

Gene symbol	Forward primer	Reverse primer
IL-1β (mouse)	*GCAACTGTTCCTGAACTCAACT*	*ATCTTTTGGGGTCCGTCAACT*
IL-1β (human)	*AGCTACGAATCTCCGACCAC*	*CGTTATCCCATGTGTCGAAGAA*
IL-6 (mouse)	*TAGTCCTTCCTACCCCAATTTCC*	*TTGGTCCTTAGCCACTCCTTC*
IL-6 (human)	*CCTGAACCTTCCAAAGATGGC*	*TTCACCAGGCAAGTCTCCTCA*
IL-1RN (mouse)	*GCTCATTGCTGGGTACTTACAA*	*CCAGACTTGGCACAAGACAGG*
IL-1RN (human)	*CATTGAGCCTCATGCTCTGTT*	*CGCTGTCTGAGCGGATGAA*
Gapdh (*mouse*)	*AGGTCGGTGTGAACGGATTTG*	*TGTAGACCATGTAGTTGAGGTCA*
GAPDH (*human*)	*GGAGCGAGATCCCTCCAAAAT*	*GGCTGTTGTCATACTTCTCATGG*

Western blot

Cells were lysed in RIPA with phosphatase and protease inhibitors. Briefly, 30 μg of protein samples were separated on 12.5 % SDS-PAGE gel and transferred to 0.45 μm polyvinylidene fluoride (PVDF) membranes. Then the membrane was blocked with 5 % non-fat powdered milk at room temperature for 1 h and incubated with the corresponding primary antibody at 4 ℃ overnight. The following antibodies were applied: IL-6 (1:1000 dilution, Beyotime, Shanghai, China), IL-1β (1:1000 dilution, Beyotime), IL-1RA (1:1000 dilution, Beyotime). At the following day, secondary antibodies were incubated at room temperature for 1 h. Protein bands were detected by ECL Western Blotting detection kit. GAPDH was used for internal control (1:1000 dilution, Huaxingbio, Beijing, China).

Rat skin wound healing experiment

The study was approved by the Animal Ethics Committee of Peking University Health Science Center (BDKQ-202511180951. SD rats (6 weeks old, male, 180±10 g) were purchased from

Beijing Vital River Laboratory Animal Technology Co, Ltd. The rats were randomly divided into two groups: negative control group (NC group) and *L. fermentum* D0S treatment group (D0S group), with 5 rats in each group. After induction anesthesia with isoflurane gas, a full-thickness skin defect wound model with a dorsal skin diameter of 10 mm was constructed in the rats. The D0S group received local application of 200 μL D0S live bacterial suspension (1×10^9 CFU/mL) daily after wound formation, while the NC group was given an equal volume of PBS. Daily observation and recording of rat body weight were performed. Images of the wound surfaces in each group were photographed on days 1, 3, 5, 7 and 9 after model construction under fixed distance and lighting conditions. All treatments were terminated on day 9, and human euthanasia procedures were performed on all experimental rats. Skin tissue from the wound and the main organs were collected. The specimens were fixed immediately in 4 % fresh-made polyformaldehyde solution for subsequent H&E staining, Masson staining, and immunostaining. The wound area was analyzed using ImageJ software, and the percentage of wound healing area was calculated using the formula: [(original wound area-current wound area) / original wound area] × 100%. All data were expressed as mean ± standard deviation. A p-value < 0.05 was considered statistically significant.

Histopathological examination

Histopathological analysis via H&E, Masson, and immunostining of IL-6, IL-1β and IL-1RA were conducted according the previous studies. For immunostaining, the IL-6 , IL-1β and IL-1RA were diluted to 1:200. The images of the stained sections were captured using the recorded using an optical microscope with digital camera.

Statistical analysis

All assays were performed in three independent experiments, each containing technical triplicates. Data are presented as the mean ± standard deviation (SD). Statistical analyses were performed by using Student t-tests or one-way ANOVA methods through GraphPad Prism software. Data were represented as mean ± SD. $p < 0.05$ was considered statistically significant.

Results

Isolation and identification of oral probiotics *L. fermentum* D0S strain

The clinical oral probiotic strains were isolated and identified according to the procedure in Figure 1A. Six oral probiotic strains were isolated and cultivated from oral cavity, and identified by sequencing the full length of 16S rRNA derived from the isolated strains after PCR. More

than 98.65% 16S rRNA gene similarity to *Limosilactobacillus fermentum* (*L. fermentum*), *Lactobacillus paracasei* (*L. paracasei*) and *Lactobacillus rhamnosus* (*L. rhamnosus*) were found after blast via NCBI Blast websites, hereafter called the two *L. fermentum* strains D0S and D1C, two *L. paracasei* strains D17A and D1D, and two *L. rhamnosus* strains D3B and D4A. The blast results of six oral probiotic strains are presented in Figure 1B and S1. In the subsequent experiments, we screened which strain exerts the best promoting wound healing in vitro and in vivo followed the flowchart in Figure 1A.

Figure 1. The oral probiotic strain isolation and identification. (A) The isolation and identification scheme. (B) The colony morphology of *Limosilactobacillus fermentum* strains D0S. (C) Blasting results for *Limosilactobacillus fermentum* D0S.

L. fermentum D0S exhibits antibacterial activity against skin wound pathogenic bacteria

To detect the inhibitory effect of the isolated oral probiotics on the skin wound pathogenic bacterial strains *S. aureus*, agar diffusion experiment was carried out. The results showed that compared with *L. paracasei* D17A and D1D, and *L. rhamnosus* D3B and D4A, *L. fermentum* D0S and D1C had the biggest inhibition zone (IZ) in live status (Figure 2B). Among the *L. fermentum* strains evaluated, D0S exhibited the most potent antibacterial activity against S. aureus. Live bacterial strain D0S and strain D1C yielded inhibition zone (IZ) of 16.5 ± 0.5 mm and 5.9 ± 0.5 mm, respectively (Figure 2C).

To objectively evaluate the antibacterial effect of D0S, we used the well-characterized antibacterial strain *L. rhamnosus* LGG as positive control and the three main skin wound pathogenic bacterial strains *S. aureus*, *S. epidermidis* and *P. aeruginosa* to reassess D0S by using agar diffusion experiments. The results showed that the living D0S produced the largest inhibition zone against *S. aureus*, followed by *S. epidermidis* and *P. aeruginosa*. Both strains exhibited slightly weaker antibacterial activity against *P. aeruginosa*. No inhibition zone was observed in the *P. aeruginosa* groups and negative control (MRS medium) groups (Figure 2D). For all the comparisons, the IZ values of the test strain D0S were statistically larger than the control strain LGG, respectively (Figure 2E-G).

Besides agar diffusion experiment, we also evaluated the anti-proliferation effect of D0S on *S. aureus*, *S. epidermidis* and *P. aeruginosa*. The growth curve results showed that the *L. fermentum* D0S had a significantly inhibitory effect on the growth of the above three pathogenic strains. Compared with the S. aureus group, the D0S + *S. aureus* group showed a lower OD_{600} value throughout the cultivation period and a longer lag phase than the *S. aureus* group (Figure 2H). The D0S + *S. epidermidis* group exhibited a significantly reduced maximum growth level

compared with the *S. epidermidis* group (Figure 2I). In the case of *P. aeruginosa*, the D0S +*P. aeruginosa* group showed a decrease in OD_{600} at the early stage of cultivation, whereas no obvious difference in total bacterial abundance was observed between the two groups at the stationary phase (Figure 2J).

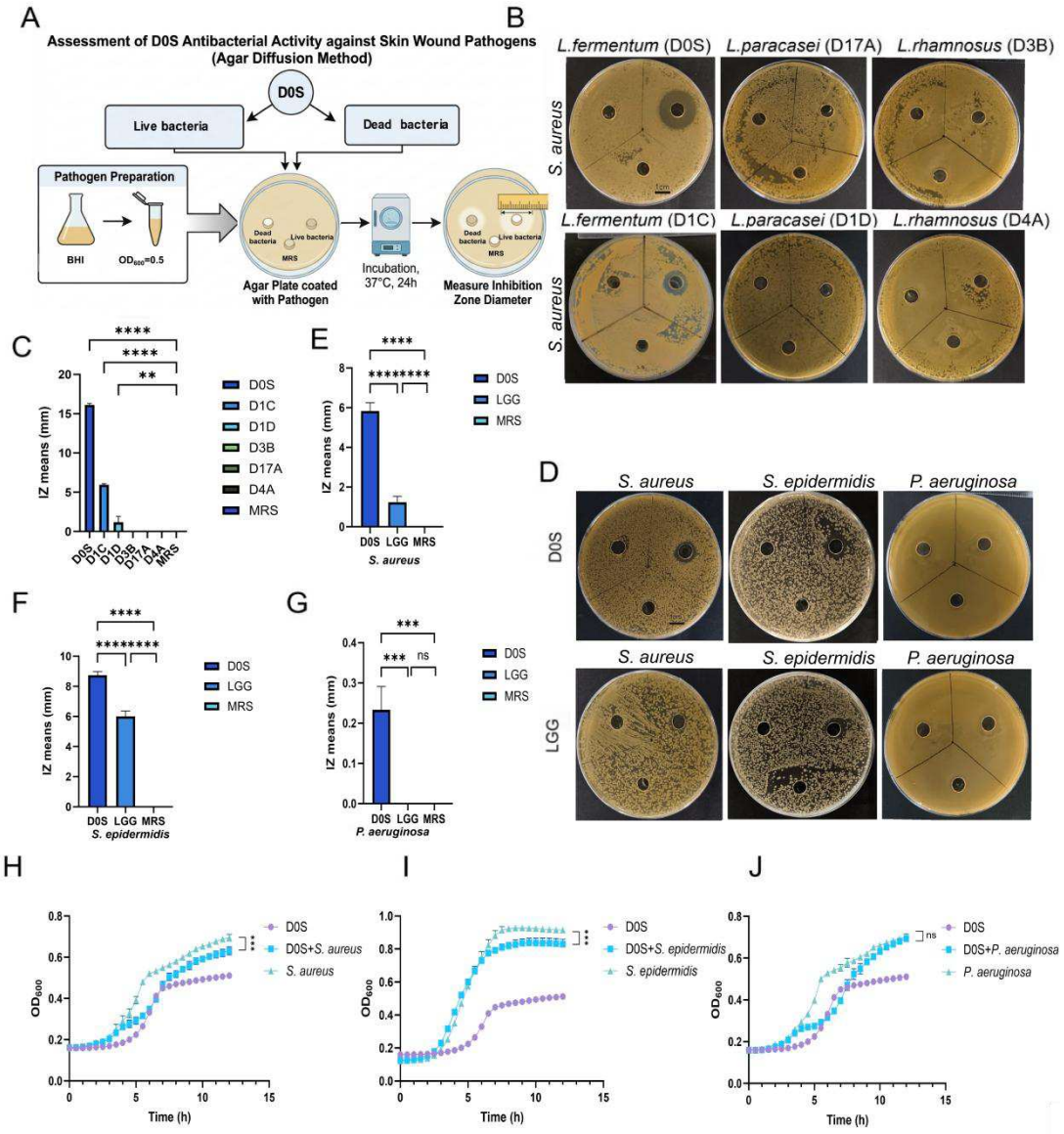


Figure 2. *L. fermentum* D0S exhibits antibacterial activity against skin wound pathogenic bacteria. (A) Schematic Representation of the agar diffusion method. (B&C) The images and statistical analysis of the diameters of inhibition zones (IZ) in *L. fermentum*-, *L. paracasei*- and *L. rhamnosus*-treated *S. aureus* groups. (D-G)The images and statistical analysis of IZ in *L. fermentum* (D0S)- and LGG-treated *S. aureus*, *S. epidermidis* and *P. aeruginosa* groups. (H-J) Growth curve of *S. aureus*, *S. epidermidis* and *P. aeruginosa* co-cultured with D0S. (ns, no significant difference; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$ at 12 h-post inoculation).

L. fermentum D0S affects the total amount of biofilms and extracellular polysaccharide content of common skin pathogenic bacteria

To investigate whether *L. fermentum* D0S can affect the formation of pathogen biofilms, we conducted a quantitative test of the biofilms using crystal violet staining. The results showed that after adding D0S to the culture system for 24 h, the biomass of biofilms by *S. aureus*, *S. epidermidis* and *P. aeruginosa* were significantly inhibited (Figure 3A-C).

Meanwhile, water-insoluble extracellular polysaccharide (EPS) content was evaluated by using the anthrone-sulfuric acid method. The results showed that D0S significantly reduced the production of EPS by the three pathogenic bacteria (Figure 3D-F).

The morphological changes in biofilm derived the D0S-treated *S. aureus*, *S. epidermidis* and *P. aeruginosa* were detected through scanning electron microscopy (SEM). The results showed that in the single culture systems of *S. aureus*, *S. epidermidis* and *P. aeruginosa*, regular, complete and smooth surfaces and dense biofilm formation could be observed (Figure 3G). After co-culture of D0S, *S. aureus* was observed to undergo bacterial atrophy, deformation, and the presence of wrinkles. The dense structure of the surface biofilm disappears or collapses completely; the cell morphology of *S. epidermidis* did not change significantly, but the dense biofilm structure became sparse and EPS disappeared. In contrast, the biofilm of *P. aeruginosa* in the single culture system was dense, but became loose after co-culture with D0S (Figure 3H). The result further verified the anti-pathogenic bacterial ability of D0S.

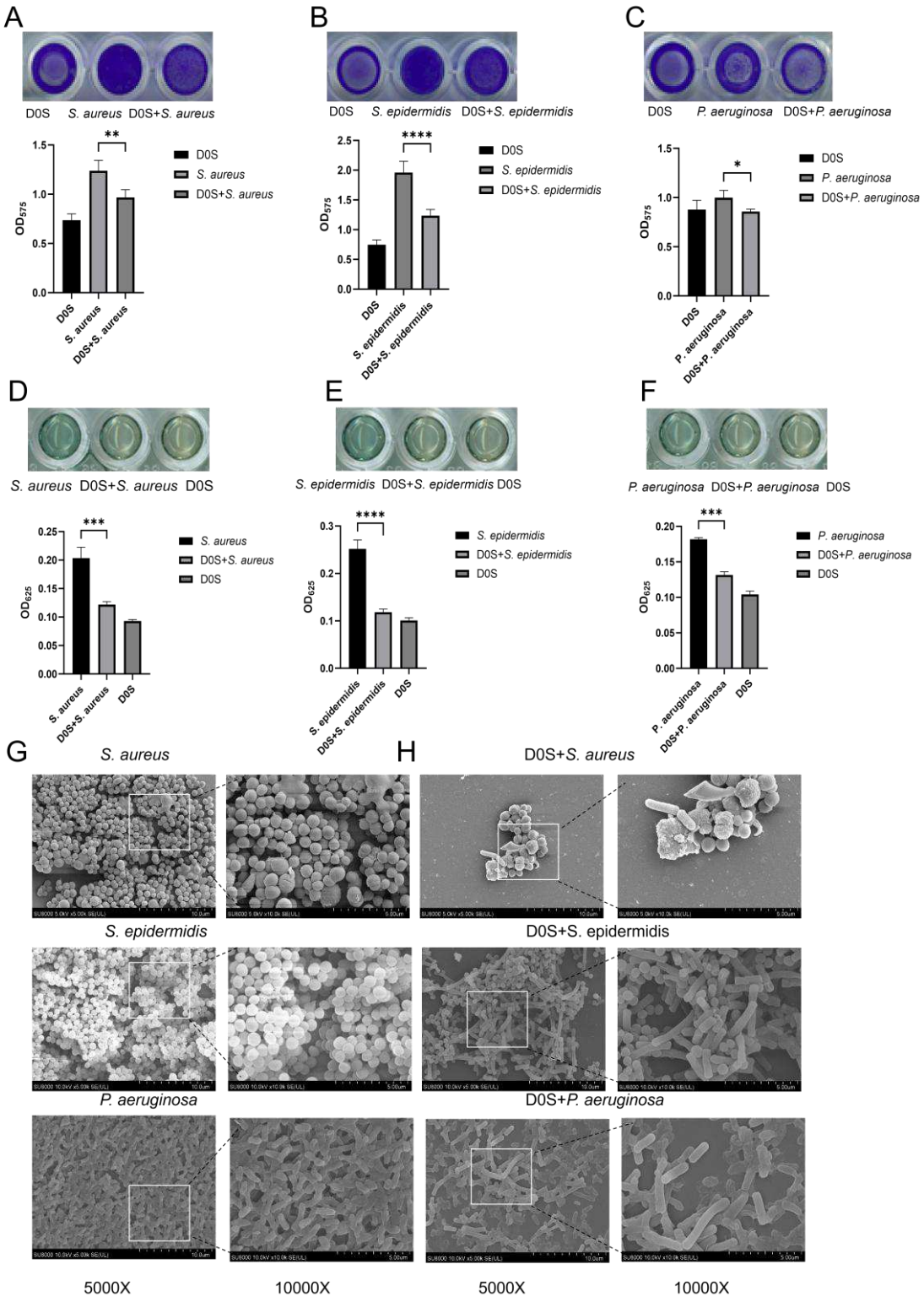


Figure 3. *L. fermentum* D0S inhibits biofilm and EPS formation abilities of *S. aureus*, *S. epidermidis*, and *P. aeruginosa*. (A-C) Images and statistical analysis of biofilm after co-culture of *S. aureus*, *S. epidermidis* and *P. aeruginosa* with D0S. (D-F) Images and statistical analysis of EPS after co-culture of *S. aureus*, *S. epidermidis* and *P. aeruginosa* with D0S. (G&H) Scanning electron microscopy (SEM) images of S. aureus, S. epidermidis and P. aeruginosa alone and co-culture with D0S. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

The effects of D0S on the migration and cell activity of eukaryotic fibroblast L929 cells and skin epithelial HaCaT cells

To assess whether the above oral probiotic strains promotes the host fibroblasts and epithelial cells migration, wound healing assay was employed by using the eukaryotic fibroblast L929 cells and skin epithelial HaCaT cells. Firstly, the optimal multiplicity of infection (MOI) of D0S was determined in D0S plus either L929 or HaCaT co-culture system through wound healing and CCK8 assays. The wound healing results showed that D0S exerted promoting migration abilities of both L929 and HaCaT cells in a dose-dependent manner, and the strongest cell migration ability was shown in MOI=10 group in L929, and MOI=50 in HaCaT, in which the scratch areas were almost completely close; while the MOI = 100 group had significantly inhibited cell migration and the scratch healing degree was the worst, even lower than that of the negative control group (Figure 4C&D). Meanwhile, CCK8 assay results showed that MOI = 100 group did affect the proliferation ability of L929 and HaCaT cells (Figure 4A&B). Therefore, we chose MOI=10 for L929 and MOI=50 for HaCaT to assess the above six oral probiotic strains through wound healing assay. The results showed that only D0S, D1C and D1D had the similar and significantly enhanced the migration rate of L929, while only D0S had the significantly enhanced the migration ability in HaCaT (Figure 4E&F).

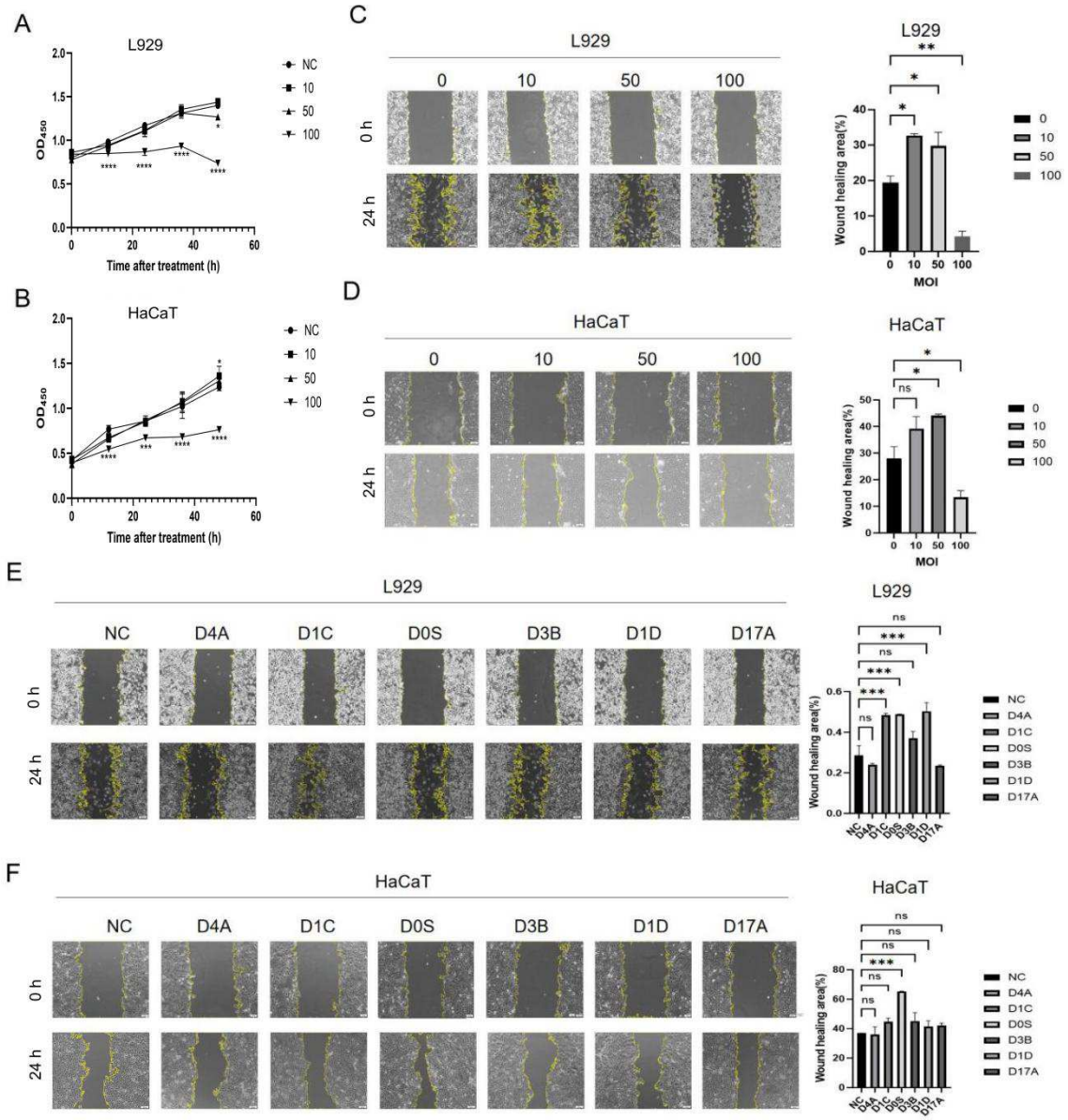


Figure 4. The effects of L. fermentum D0S on the migration and cell activity of eukaryotic fibroblast L929 cells and skin epithelial HaCaT cells. (A&B) The CCK8 method was used to detect the viability of L929 cells and HaCaT cells after co-culturing with different MOI values of D0S. (C&D) Effects of D0S under different MOI values of L929 and HaCaT cells on the migration ability. (E&F) Wound healing assay and statistical analyses of L929 cells and HaCaT cells after co-culturing with optimized MOI of six oral probiotic strains. (ns, no significant difference; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

D0S has slightly higher the promoting proliferation ability of epithelial cells than LGG

Using the standard strain *L. rhamnosus* LGG as control, we further evaluated the the value of D0S in promoting skin wound healing. The results showed that the MOI=10 D0S and LGG exhibited similar cell proliferation and migration rates in L929 (Figure 5A&B). In the HaCaT cell co-culture system, MOI=50 D0S group exhibited similar migration rate but slightly higher proliferation ability than LGG at 24 h post-scratch (Figure 5C&D).

D0S decreases pro-inflammatory factor expression but increases anti-inflammatory mediator IL-1RN expression in fibroblasts and epithelial cells

To investigate whether D0S has the ability to regulate immunity mediator expression, we did qPCR and western blot assays in D0S-treated L929 and HaCaT cells. The results showed D0S decreased pro-inflammatory cytokines IL-1β and IL-6 expression but increases an anti-inflammatory mediator IL-1RN expression in fibroblast L929 and epithelial HaCaT cells through qPCR. IL-1RA , encoded by IL-1RN, was also detected using western blot, which was in line with qPCR's results (Figure 5E) .

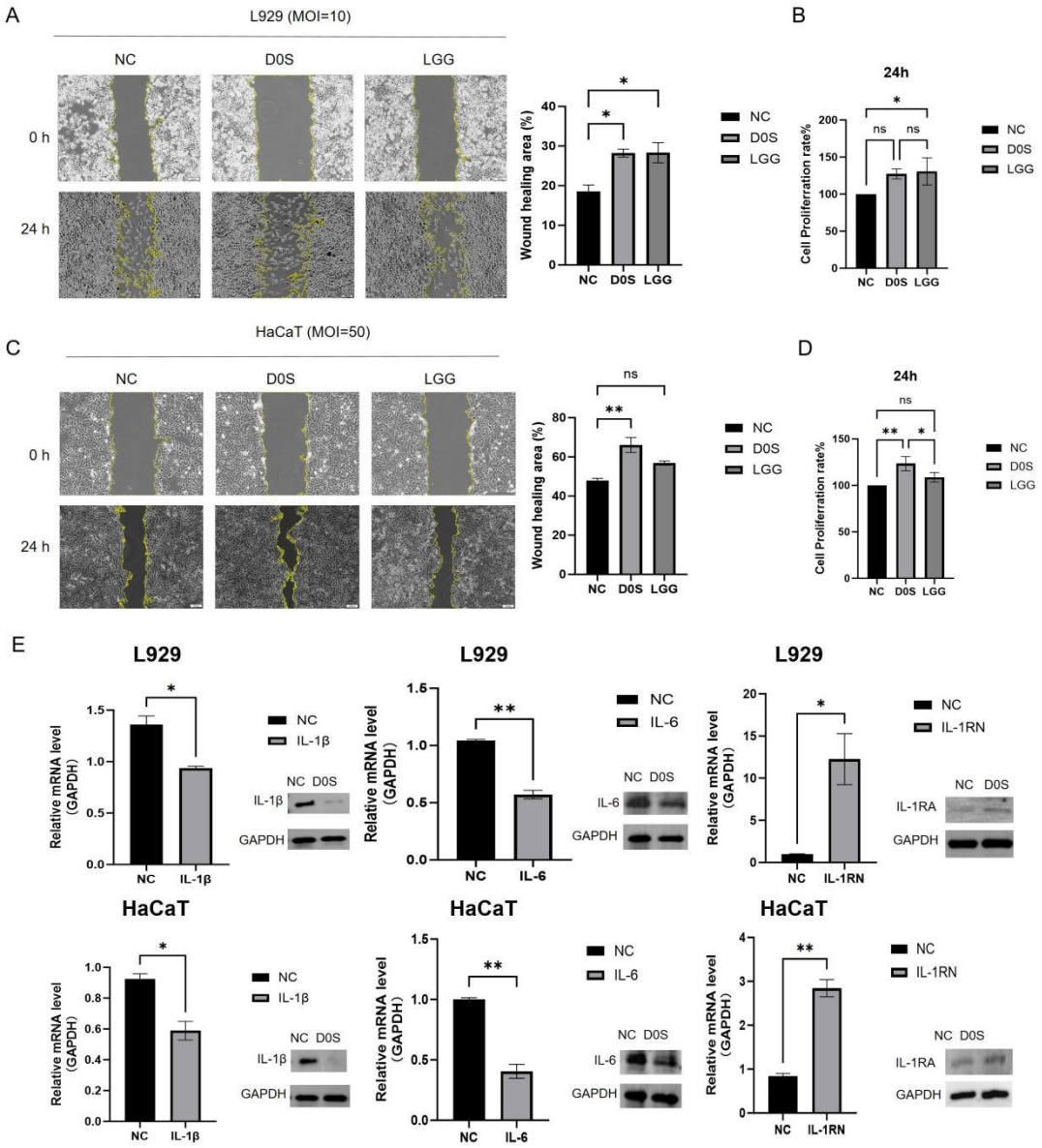


Figure 5. Comparison of the effects of D0S and LGG on modulating inflammatory mediates expression and promoting proliferation and migration abilities in fibroblasts and epithelial cells. (A&B) Effects of D0S and LGG of L929 cells on the migration and proliferation abilities of L929 cells. (C&D) Effects of D0S

and LGG of HaCaT cells (MOI=50) on the migration and proliferation abilities of HaCaT cells. (E)IL-1β, IL-6 and IL-1RN expression of D0S-treated L929 and HaCaT cells.(ns, no significant difference; *, $p < 0.05$; **, $p < 0.01$).

D0S accelerates skin wound healing in vivo

To evaluate whether D0S promotes skin wound healing in vivo, we established a diameter of 10 mm full-thickness skin wound model on the back of rats, and treated by D0S followed the procedure in Figure 6A. The results showed that the D0S group exhibited more pronounced wound contraction starting from day 3, with significantly faster healing rates than the NC group. By day 5, the wound area in the D0S group had markedly reduced. By day 9, the wound in the D0S group was nearly healed (Figure 6B) which indicated that during the intermediate phase of wound healing (days 3‑5), D0S significantly accelerated wound healing rates (Figure 6C). During the period of the experiments, the monitoring results of body weight changes (Figure S2) showed from the second to the seventh day except the fourth day after the injury, the weight of rats in the D0S group increased significantly faster than that in the NC group. The result indicates that D0S treatment can promote the overall recovery of rats after trauma, possibly related to reducing pain, inflammatory response, and stress state.

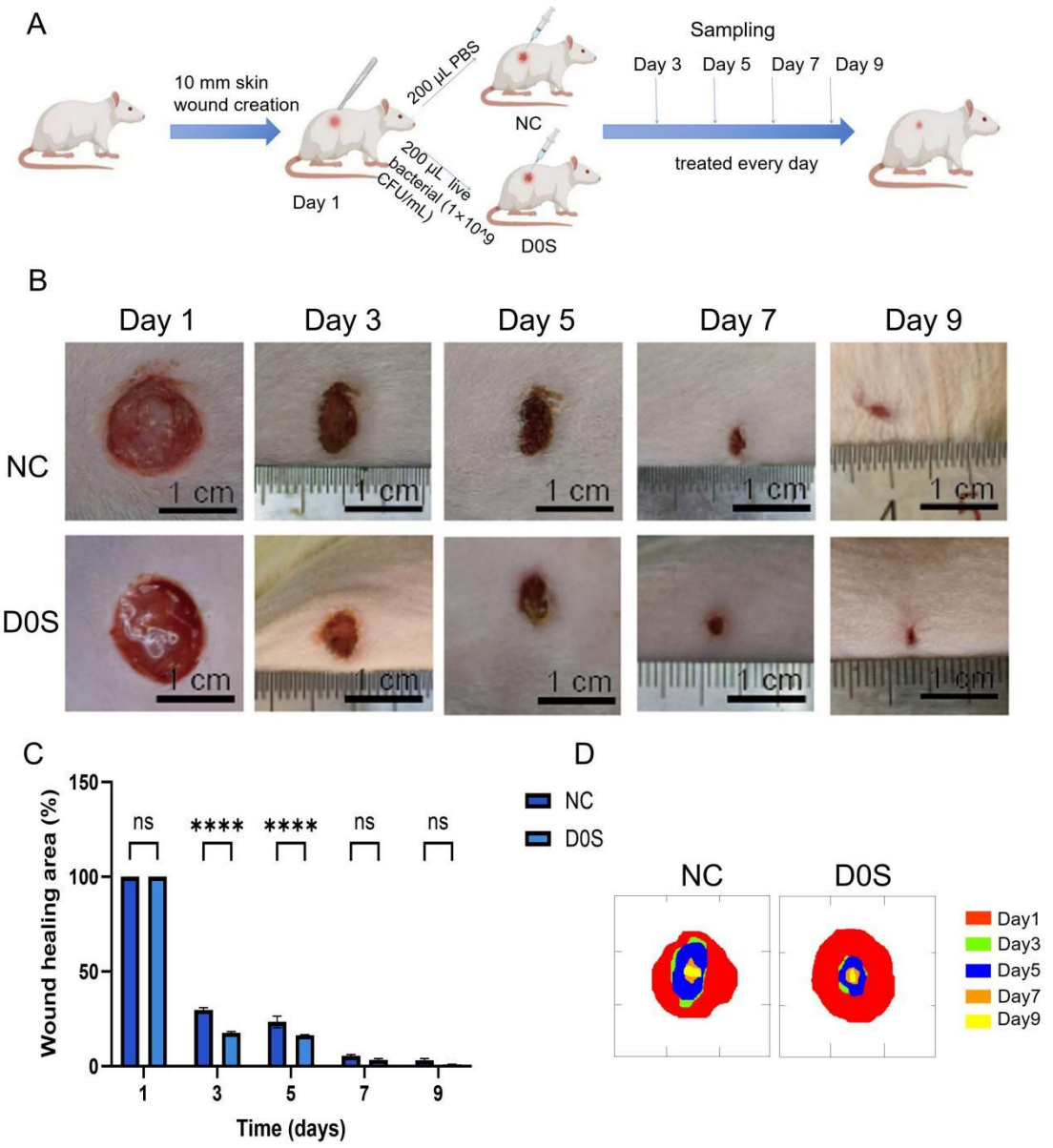


Figure 6. D0S accelerates skin wound healing in vivo. (A) Animal experiment scheme. (B) Images of wounds at various time points in each group over 9 days. (C) Quantitative analysis of wound area at different time points in each group. (D) Unhealed wound areas on days 1, 3, 5, 7 and 9 (ns, no significant difference; ****, $p < 0.0001$).

Histopathological examination reveals that D0S accelerates skin wound healing through improving immune microenvironment and promoting collagen deposit and re-epithelialization

To verify whether D0S promotes skin wound healing through modulating immune microenvironment surrounding the wound, we performed a histopathological examination by using skin wound healing tissues and the main organ tissues through H&E staining, Masson's trichrome staining and immunohistochemical staining. H&E staining results showed that the

D0S group exhibited markedly enhanced epidermal regeneration compared with the NC group, with clear evidence of granulation tissue formation and re-epithelialization (Figure 7A). Specifically, the D0S group displayed a higher degree of re-epithelialization, increased epidermal thickness and an accelerated wound contraction rate .

Wound healing depends on the deposition of collagen, which is evaluated by Masson's trichrome staining. The results showed that compared with the NC group, the collagen deposition in the D0S group was higher, showing a denser, more uniform and systematic arrangement of thick collagen, while the collagen arrangement in the control group was relatively loose. These results indicate the efficiency of D0S in treating skin wounds (Figure 7B).

IL-1β and IL-6 are pro-inflammatory cytokines that play a key role in the immune response and are often used as markers of inflammation and infection. This study evaluated the relative levels of IL-1β and IL-6 in each group through immunohistochemical analysis (Figure 7C). Compared with the NC group, the expression of IL-1β and IL-6 in the D0S group was relatively reduced. In addition, IL-1RA is a natural anti-inflammatory cytokine in the IL-1 family that regulates inflammatory responses by competitively inhibiting the IL-1 signaling pathway. The expression level of IL-1RA in the D0S group was higher than that in the NC group on the day 9 (Figure 7C), suggesting the inhibition of the inflammatory response. Consistent with the results of in vitro experiments, these results indicated that local application of D0S can promote the transformation of tissue cells to a less inflammatory and more reparative phenotype.

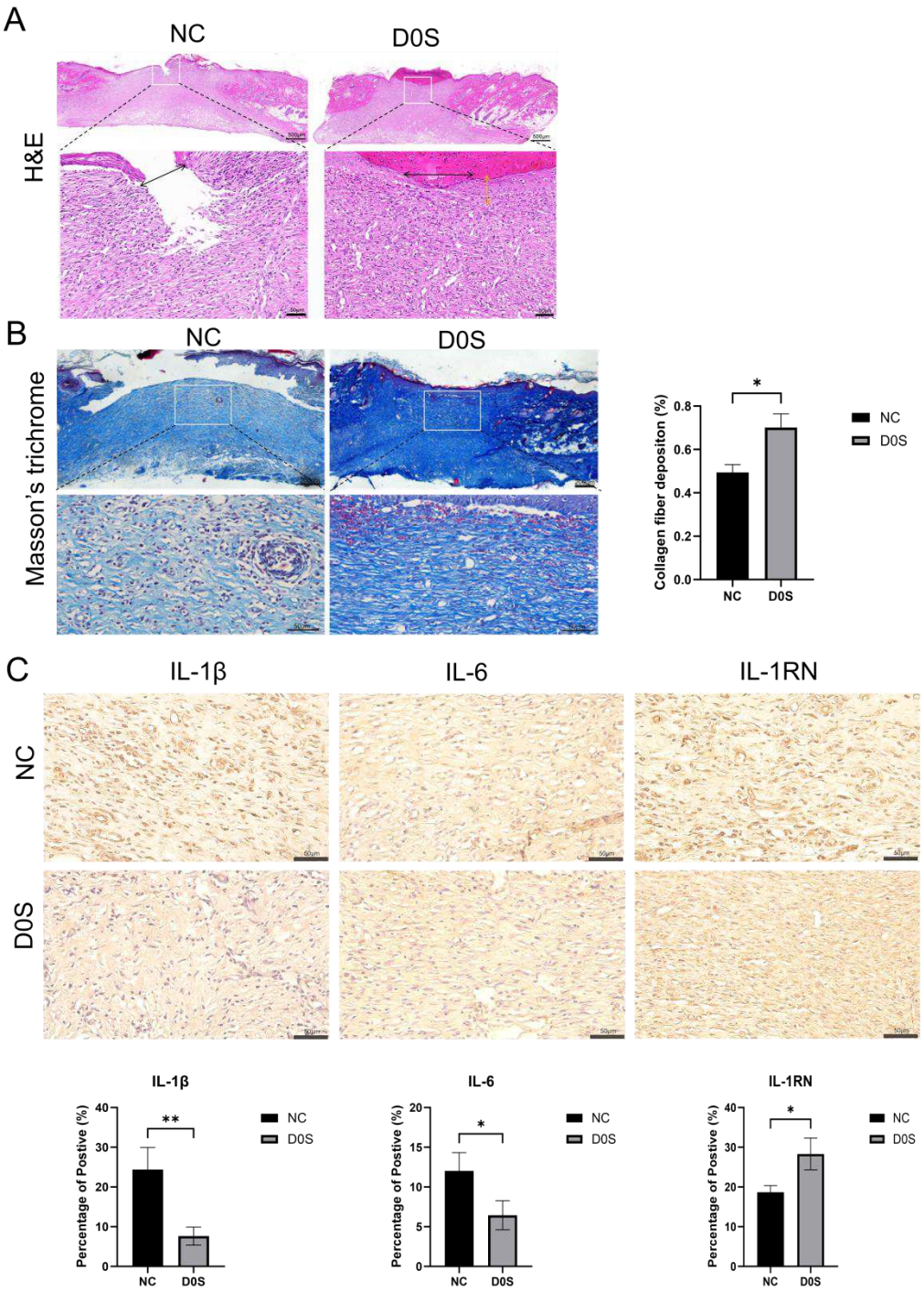


Figure 7. Histopathological examination to assess the effect of D0S on skin wound healing in rat. (A)H&E stained images of skin tissue,which shows the D0S group displayed a higher degree of re-epithelialization, increased epidermal thickness (indicated by the yellow arrow), and an accelerated wound contraction rate (indicated by the black arrow). (B) Masson's trichrome staining image of skin tissue. (C) Immunohistochemical staining and statistical analysis of IL-1β, IL-6 and IL-1RA (*, $p < 0.05$; **, $p < 0.01$).

Discussion

In this study, we isolated a novel oral probiotic strain *L. fermentum* D0S, which showed a better promoting skin wound healing rate than a well-known probiotic strain *L. rhamnosus* LGG. Compared with LGG, *L. fermentum* D0S shows the stronger antibacterial activity, higher promoting epithelial cell proliferation, and enhanced immunomodulatory ability during the process of skin wound healing in vitro and in vivo, indicating the potential use in skin wound healing.

Our results demonstrated that *L. fermentum* D0S significantly promoted wound healing at three levels : pathogen level, host cell level and immunomodulatory level. First, at the pathogen level, we choose three common pathogens of skin wound, *S. aureus*, *S. epidermidis* and *P. aeruginosa*. Many studies show that pathogen amount, biofilm and EPS are the important virulent factors, which determined the severity of wound infection(Demir & Aslim, 2025). A decrease in the amount of pathogens, biofilm and EPS markedly alleviates the severity of wound infection, just creating a microenvironment conducive to wound healing. Second, at the host cell level, considering skin has epithelial (epidermis, dermis), and subcutaneous tissue (main cell component is fibroblasts) layers, we choose fibroblast cell line L929 and immortalized human epithelial cell line HaCaT as our models. We find D0S can not only stimulate L929 proliferation and migration, but also express collagen to strength the skin wound's resistance to splitting and to cause the wound to contract and shrink. We infer that these are the key factors promoting early wound healing. Epithelial tissue is an important defense and protective barrier of the skin (Madison, 2003). *L. fermentum* D0S can promote faster epithelial cell HaCaT proliferation and migration than LGG. This indicates epithelial cells repair and close wounds faster, just providing a protective effect. Third, at immunomodulatory level, this study revealed that D0S enhances skin wound healing by down-regulating pro-inflammatory cytokines IL-1β and IL-6, and up-regulating anti-inflammatory mediator IL-1RN/IL-1RA. Kou et.al showed that MSCs produce IL-1RA to maintain rapid wound healing in the gingiva, which is in line with our study(Kou et al., 2018). Wang et.al showed that bacteria induce skin regeneration via IL-1β signaling (Wang et al., 2021). In addition, the immunomodulatory function of probiotics is also consistent with previous findings (Silva et al., 2018), in which Lactobacillus strains facilitate tissue regeneration and differentiation during the early stages of wound healing by reducing pro-inflamatory cytokine expression(Kim et al., 2020), remodeling the extracellular matrix and promoting fibroblast migration (Chen et al., 2024). These hypotheses were therefore verified in rat skin wound healing experiments in this study.

Conclusions

In summary, our results support new isolated *L. fermentum* D0S is marginally superior to LGG in promoting skin wound healing function. D0S can exert its therapeutic effect in skin wound microenvironment rather than oral cavity microenvironment. Mechanistically speaking, D0S not only inhibits the proliferation in the three common pathogenic bacterial strains *S. aureus*, *S. epidermidis* and *P. aeruginosa*; down-regulates pro-inflammatory cytokines IL-1β and IL-6, up-regulates anti-inflammatory mediator IL-1RA expression, but also promotes collagen deposition in skin wounds, creating a faster and more stable environment for tissue repair. The shortcoming of this article lies in the insufficiently in-depth research on mechanisms and potential applications of complex and infected wounds. Future research is needed.

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Author Contributions

Conceptualization, Y.W.; methodology, Y.H., S.L and J.Z.; validation, Y.C., J.C. and J.S.; formal analysis, Y.H., S.L; investigation,Y.H., S.L; writing—original draft preparation, Y.H.; writing—review and editing, Y.W.; supervision, Y.W. and X.G.; project administration, Y.W.,C. D.; funding acquisition, YW. All authors have read and agreed to the published version of the manuscript.

Supporting material

Supplementary material is available at Journal of Applied Microbiology online.Figure S1: The blast results of *L. fermentum* srtains D1C, two *L. paracasei* strains D17A and D1D, and two *L. rhamnosus* strains D3B and D4A. Figure S2. The rat body weight changes during the 9-day animal experiment.

Conflicts of Interest

The authors have no conflicts of interest to declare.

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Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval

The study was approved by the Animal Ethics Committee of Peking University Health Science Center (BDKQ-202511180951.

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