Supplementary Information

Magneto-catalytic Biohybrid Bacterial Bots Efficiently Eradicate and Remove Biofilm in Catheter

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Materials and methods

Fabrication of Magnetic bacterial bots: *L. rhamnosus* (*L. rhm*) bacteria MTCC-1408 in their late log phase were utilized to fabricate magnetic bacterial bots.[1] The process involved two steps in which (a) gold nanoclusters bearing *L. rhm* bacteria (Au-Lac) were synthesized, followed by (b) attachment of quercetin-loaded magnetic nanoparticles over the Au-Lac surface (QM-Au-Lac) as described in the reported protocol. In brief, 2 mM of gold solution ($HAuCl_4$) had been reduced with a mild reducing agent, 0.11 M 3-mercaptopropionic acid (MPA), which was introduced to the bacterial aqueous dispersion having a bacterial concentration of 10^9 CFU/mL. Further, quercetin-loaded magnetic nanoparticles (QMNPs) were incubated with 7.5 mM of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) for 15 min, followed by incubation with 7.5 mM N-hydroxysuccinimide (NHS) for 15 min at 37 °C. The activated solution of QMNPs was added to the Au-Lac pellet and kept at 37 °C. After 15 min, through an external magnet, magnetic QM-Au-Lac was separated, followed by washing twice with autoclaved deionised water with centrifugation at 3000 rpm for 5 min.

Methylene blue degradation assay: Based on the idea that ·OH can break down methylene blue (MB) and thus, reduce its absorbance at 663 nm, ·OH was probed by performing the MB degradation assay. In 2.7 mL of MB solution (8 μg/mL), 0.3 mL each of various treatment groups was added and incubated in the dark for 1 h. The treatment group includes quercetin, QMNPs, Lac, Au-Lac, and QM-Au-Lac. Since 250 μM of quercetin was loaded into magnetic

bacterial bots, so the same concentration of free quercetin and QMNPs was used in the assay. Also, the bacterial concentration was kept at 5 x 10^5 CFU/mL throughout the experiment. In the case of light-irradiated samples, they were irradiated with red LED light at 632 nm wavelength (0.5 W/cm^2) for 10 min.

After 1 h, the supernatant was collected by centrifuging the mixture at 5000 rpm for 10 min. The absorbance of the collected supernatant of each treatment group at 663 nm was recorded using a Shimadzu UV-Vis-spectrophotometer.

Rhodamine B degradation assay: To check the photocatalytic activity of QM-Au-Lac, rhodamine B (RhB) dye was used. For the experiment, in 0.9 mL of RhB solution (25 mg/L), 0.1 mL of each of the various treatment groups (in separate containers) was added and incubated in the dark for 1 h. The treatment groups were similar to the MB degradation assay mentioned above. The light-irradiated samples were exposed to light (of 632 nm, 0.5 W/cm^2) for 10 min.

After the completion of 1 h, the samples were centrifuged at 5000 rpm for 10 min. The photoluminescence (PL) spectra of the collected supernatant were taken using a Thermo Scientific multiplate reader at 600 nm wavelength.

Glutathione (GSH) depletion assay: To evaluate the GSH depletion ability of the magnetic bacterial bots, the 5,5′-dithio-bis-2-nitrobenzoic acid (DTNB) reagent was used to perform the GSH depletion assay. In brief, 1 M of GSH solution was prepared freshly in HAc-NaAc buffer of pH 5.5. In 1 mL of GSH solution, 100 μL of each of the treatment group was added in the dark. From that, 500 μL was taken, and 25 μL of DTNB (20 mM in freshly prepared Hac-NaAc buffer of pH 7.4) was added and kept in the dark for 1 h. In the case of light-irradiated samples, they were irradiated with light (of 632 nm, 0.5 W/cm^2) for 10 min.

After 1 h, the supernatant was collected by centrifuging the mixture at 5000 rpm for 10 min. The UV-Vis spectra of the collected supernatant of each of the treatment groups at 412 nm wavelength were recorded using a Shimadzu UV-Vis-spectrophotometer, and the O.D. of the same samples were taken using a Glomax multi-plate reader.

Photocatalytic activity of QM-Au-Lac: The photocatalytic activity of QM-Au-Lac was examined against both MB and RhB dye. For this, 0.1 mL of QM-Au-Lac was added to 0.9 mL of MB solution (8 μg/mL) and RhB solution (25 mg/L) in the dark for 1 h. In the case of light-irradiated samples, they were irradiated with light (0.5 W/cm^2) for 10 min first, followed

by incubation for 1 h. After every 10 min, samples were centrifuged, and the supernatant was collected. Using the GloMax plate reader, the absorbance of the MB solution was measured at 600 nm. For the RhB solution, the PL intensity was recorded by using an excitation wavelength of 520 nm and an emission wavelength of 540-580 nm. To calculate the degradation percentage of MB and RhB, equations (1) and (2) were used, respectively.

MB Degradation% = $[(Abs_0-Abs_t)/ Abs_0] \times 100$, where Abs_0 is the initial absorbance of MB and Abs_t is the absorbance at a particular time interval (1)

RhB Degradation% = $[(PL_0-PL_t)/ PL_0] \times 100$, where PL_0 is the initial PL intensity of RhB and PL_t is the PL intensity at a particular time interval (2)

The pseudo-first-order rate constant (k) for the degradation of dyes was calculated using the Langmuir-Hinshelwood equation.

$$\ln(C_t/C_o) = -k \times t \quad (3)$$

where C_o and C_t denote the initial concentration and reaction concentration, respectively.

Anti-biofilm formation assay: In a 96-well plate, the biofilms of two pathogenic bacteria, i.e., *S. aureus* (MTCC 96) and *P. aeruginosa* (MTCC 2488), were formed. For this, 0.25% dextrose-containing Brain Heart Infusion (BHI) media and Nutrient Broth (NB) media were used for the formation of *S. aureus* and *P. aeruginosa* biofilm, respectively. In each well, 200 µL of bacteria ($1x10^6$ cfu/mL) was added and incubated for 24 h in the static incubator at 37 °C. After 24 h, the media containing dextrose was discarded carefully without disrupting the biofilm. The biofilm was further treated with media-containing treatment groups for 24 h. The treatment group includes quercetin, QMNPs, Lac, Au-Lac, and QM-Au-Lac. Since 250 µM of quercetin was loaded into magnetic bacterial bots, so the same concentration of free quercetin and QMNPs was used in the assay. Also, the bacterial concentration (Lac, Au-Lac, and QM-Au-Lac) was kept at 5×10^5 CFU/mL throughout the experiment. To precisely determine the viability without being influenced by the live bacteria of the treatment groups, the crystal violet test was carried out with treatment groups devoid of biofilm. In the case of light-irradiated samples, they were irradiated with red LED light at 632 nm wavelength (0.5 W/cm^2) for 10 min.

The biofilm after 6 h of treatment was irradiated with LED light of 632 nm wavelength (for 10 min at 0.5 W/cm^2). Further, the media was discarded with PBS twice to remove unadherent

bacteria, and 0.1% crystal violet was added for 30 min and then dissolved using absolute ethanol. The absorbance reading was measured at 600 nm using a Glomax plate reader.

Live-dead imaging of biofilm using confocal microscopy (CLSM): In a 96-well plate, biofilms of *S. aureus* and *P. aeruginosa* were formed and treated after light irradiation using the same method as mentioned above. Further, the biofilms were washed and stained with acridine orange (10 μg/mL) and propidium iodide (10 μg/mL) for 15 min, followed by imaging. To discriminate between live bacteria, which fluoresce green, and dead cells, which fluoresce red samples were exposed to 488 and 514 nm lasers, respectively.

Surface and morphological characterization: For field emission scanning electron microscopy (FESEM) imaging, biofilm samples were prepared on the coverslip, which was air-dried overnight, and images were recorded using the Jeol JSM 7610 FESEM instrument.

Evaluation of ROS generation: To determine the ROS generated by the treated biofilm, 2′,7′-dichlorodihydrofluoroescein (DCFDA) dye was used. Firstly, biofilms were formed in a 96-well plate and then treated with the treatment groups at different time intervals. After treatment, the light was irradiated for 10 min, and DCFDA dye (15 μM) was added to each well and incubated for 30 min in the dark. Further, in a Glomax plate reader, 475 nm wavelength was used for excitation and 500-550 nm for emission to take the measurement. To determine ROS generation precisely, treatment groups incubated without the biofilms were also kept to avoid the influence of live bacteria (Lac, Au-Lac, and QM-Au-Lac).

For CLSM imaging, biofilm was grown in a confocal dish, followed by treatment, and then imaging was carried out using a 488 nm laser.

Assessment of lipid droplet content:- To evaluate the intracellular lipid droplet content of bacterial biofilm, Nile red dye was used. The biofilms were grown in a 96-well plate and then treated with the treatment group for 24 h. The biofilm after 6 h of treatment was irradiated with LED light of 632 nm wavelength (for 10 min at 0.5 W/cm^2). After 24 h, the biofilms were stained with Nile red dye (0.01mg/mL) for 30 min and then washed with PBS carefully. The change in lipid droplet content was quantified by evaluating the PL intensity of samples that were excited with a 520 nm wavelength and at the emission measured at 580-640 nm wavelengths in the Glomax plate reader.

For CLSM imaging, the biofilm was grown in a confocal dish, followed by treatment, and then imaging was carried out using a 488 nm laser.

Lipid peroxidation assay:- Lipid peroxidation of bacterial biofilm was determined using BODIPY C11 dye. In a confocal dish, 10^6 CFU/mL, *S. aureus/ P. aeruginosa* were added and incubated for 24 h to form biofilm. Further, biofilms were treated with QM-Au-Lac (after 6 h, irradiated with light for 10 min, as appropriate) for the next 24 h. After 24 h, the biofilms were stained with BODIPY C11 dye (5 μM) for 30 min, and then imaging was carried out using CLSM.

Biofilm eradication in a urethral catheter:- To study the effectiveness of magnetic bacterial bot, a catheter was used as an *in vitro* model. *P. aeruginosa* biofilm was prepared in the catheter pipe that was cut into pieces of 10 cm. In a catheter pipe, 10^6 CFU/mL, bacterial suspension was carefully added and allowed to incubate for 48 h. After 48 h, the plankton cells were washed out, and QM-Au-Lac solution (10^6 CFU/mL) was carefully added, followed by incubation for 24 h. The sample was stained with 0.1% crystal violet to visualize the effectiveness of QM-Au-Lac. For FESEM imaging, the part of the catheter was cut into 1 cm and was fixed with 2.5% glutaraldehyde, followed by PBS wash, and the catheter was air dried. For confocal imaging, the catheter was stained with propidium iodide (10 μg/mL) and acridine orange (10 μg/mL) for 30 minutes, followed by PBS wash and fixing, and then the catheter was analysed in CLSM.

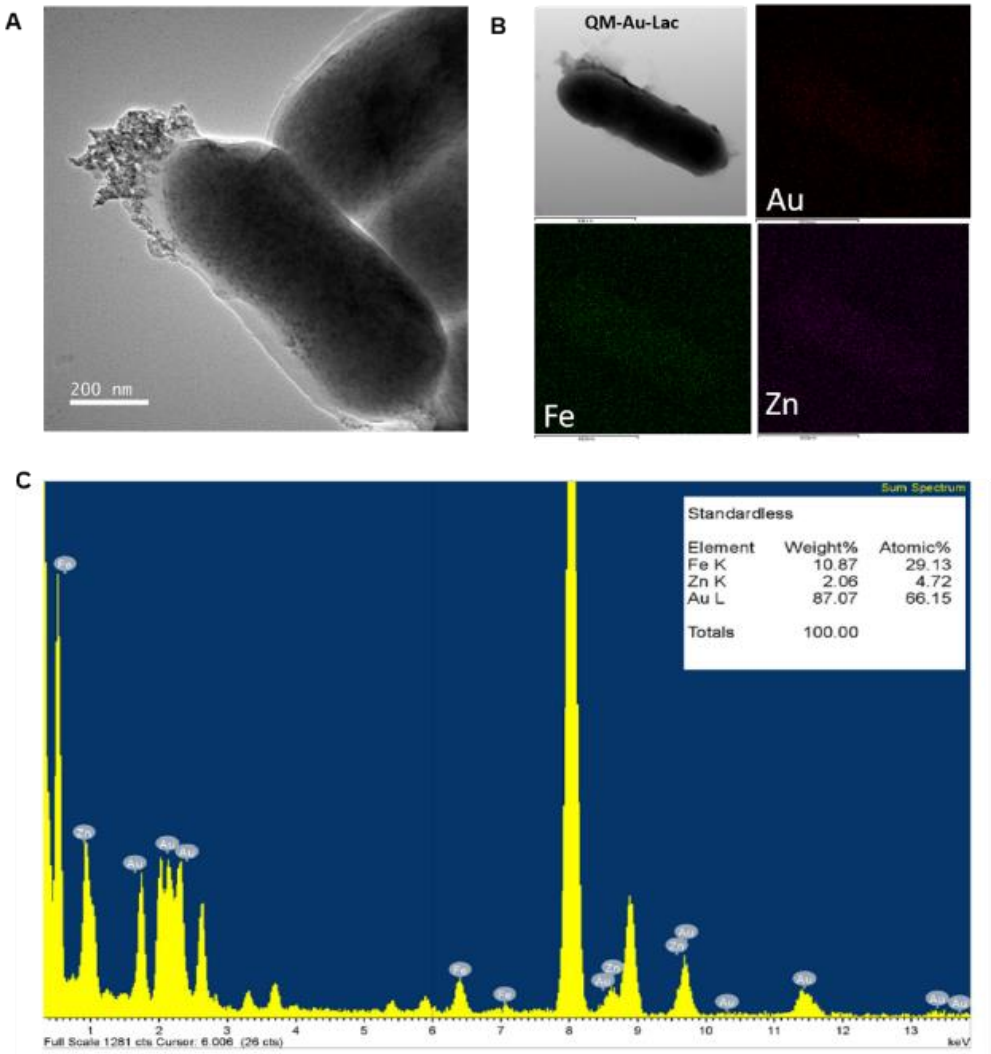

Fig. S1 (A) FETEM image of QM-Au-Lac. (B) Elemental mapping and (C) Energy-dispersive X-ray spectrum of QM-Au-Lac.

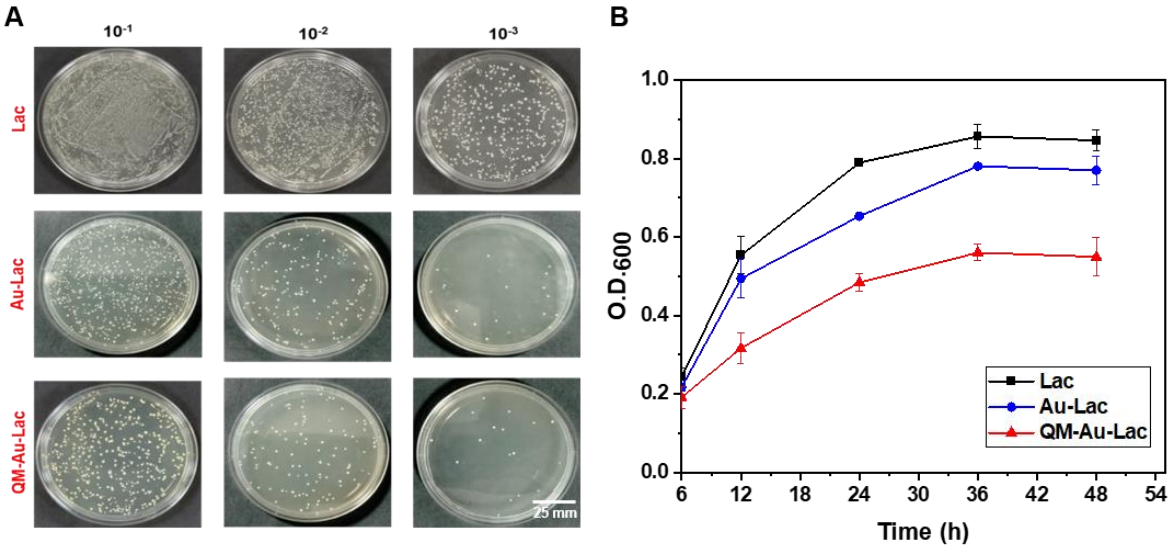

Fig. S2 (A) Images of the MRS-agar plate used for CFU (colony forming unit/mL). The initial concentration of bacteria used for synthesis was 10^9 CFU/mL. The Lac, Au-Lac, and QM-Au-Lac were plated after being irradiated with red light (of 630 nm, 0.5 W/cm^2) for 10 minutes. Scale bar is 25 mm (B) Growth curve of Lac, Au-Lac, and QM-Au-Lac for 48 h after being irradiated with red light (of 630 nm, 0.5 W/cm^2) for 10 minutes.

Table S1 Table representing the percentage of MB degradation after treatment with different treatment groups as mentioned in the legends. The results have been calculated based on the graphs in Fig. 1C.

MB treated with	Without light	With light
H_2O_2 (1mM)	18.02±0.1	18.25±1.5
Quercetin	31.27±3	37.78±0.8
QMNPs	39.29±1.3	45±1.1
Lac	20±0.9	19.21±1.8
Au-Lac	20.56±0.1	18.81±0.8
QM-Au-Lac	82.78±0.4	86.11±0.8

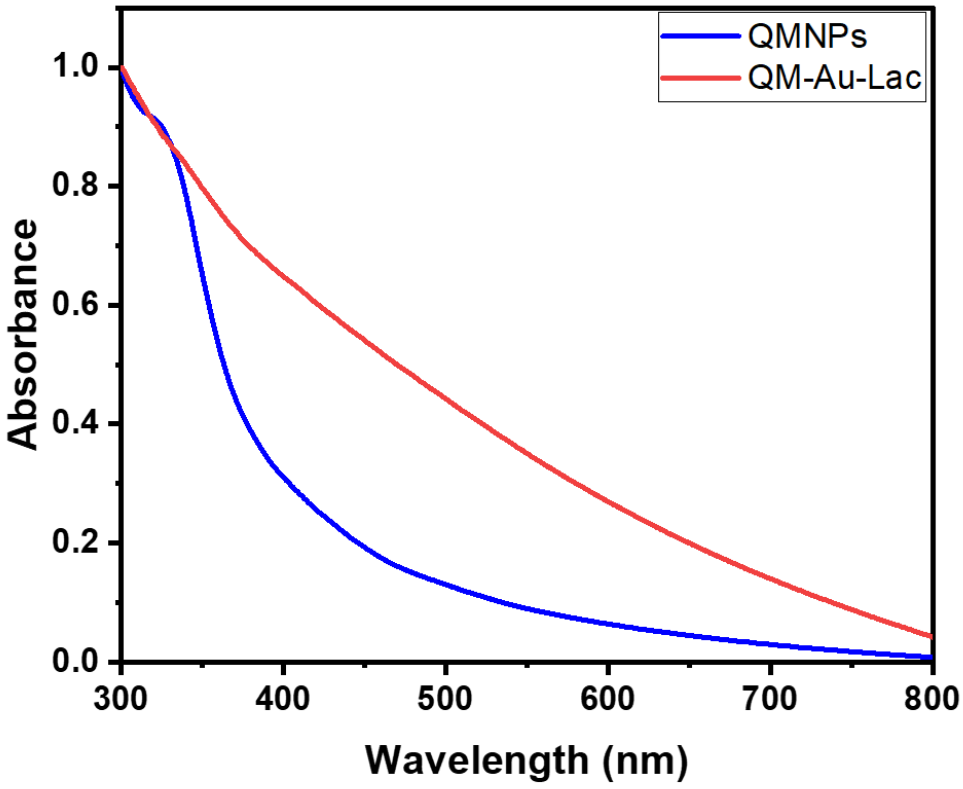

Fig. S3 UV-Vis absorption spectra of QMNPs and QM-Au-Lac.

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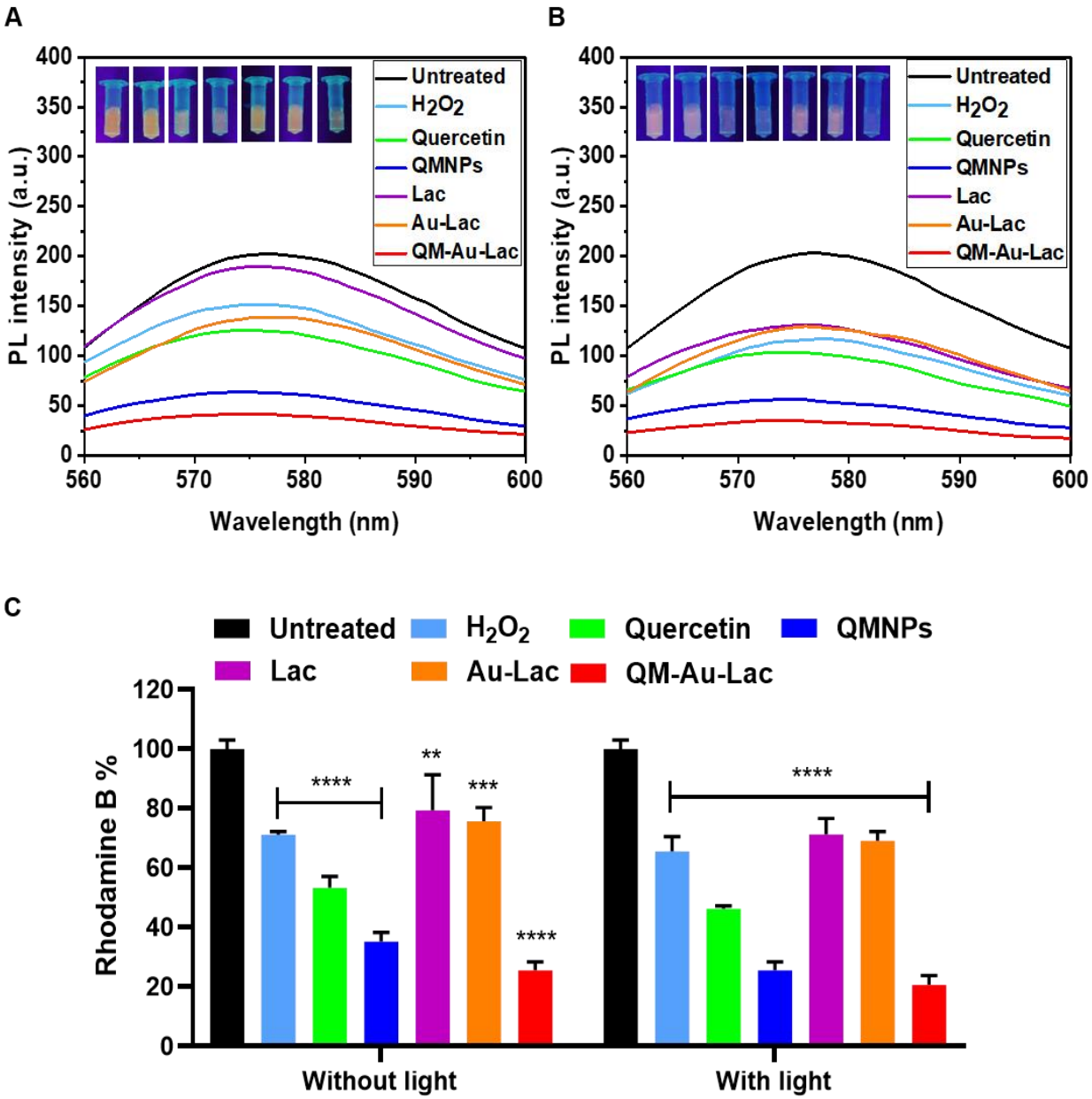

Fig. S4 PL spectra of RhB treated with 1 mM H_2O_2 (positive control), quercetin, QMNPs, Lac, Au-Lac, and QM-Au-Lac (A) without light and (B) with light irradiation. (C) Graph representing the percentage of RhB in the samples. Statistical significance is represented by ** ($p<0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). The values are represented as mean ± standard deviation of three individual experiments

Table S2 Table representing the percentage of RhB degradation after treatment with different treatment groups as mentioned in the legends.

RhB treated with	Without light	With light
H_2O_2 (1mM)	28.09±1.1	34.53±5.0
Quercetin	46.75±3.9	53.87±1.0
QMNPs	64.81±3.0	74.58±2.9
Lac	20.73±11.9	28.81±5.3
Au-Lac	24.30±4.5	30.96±3.1
QM-Au-Lac	74.58±2.9	79.49±3.2

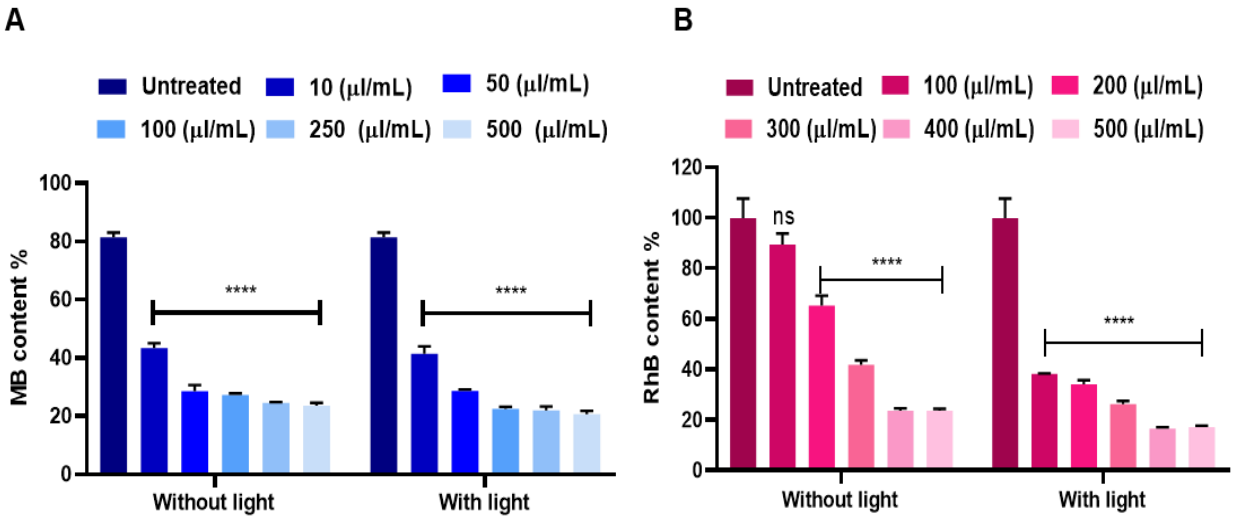

Fig. S5 Graph representing the (A) MB content percentage, and (B) RhB content percentage after treating with different concentrations of QM-Au-Lac for 1 h. Statistical significance is represented by ** ($p<0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). The values are represented as mean ± standard deviation of three individual experiments.

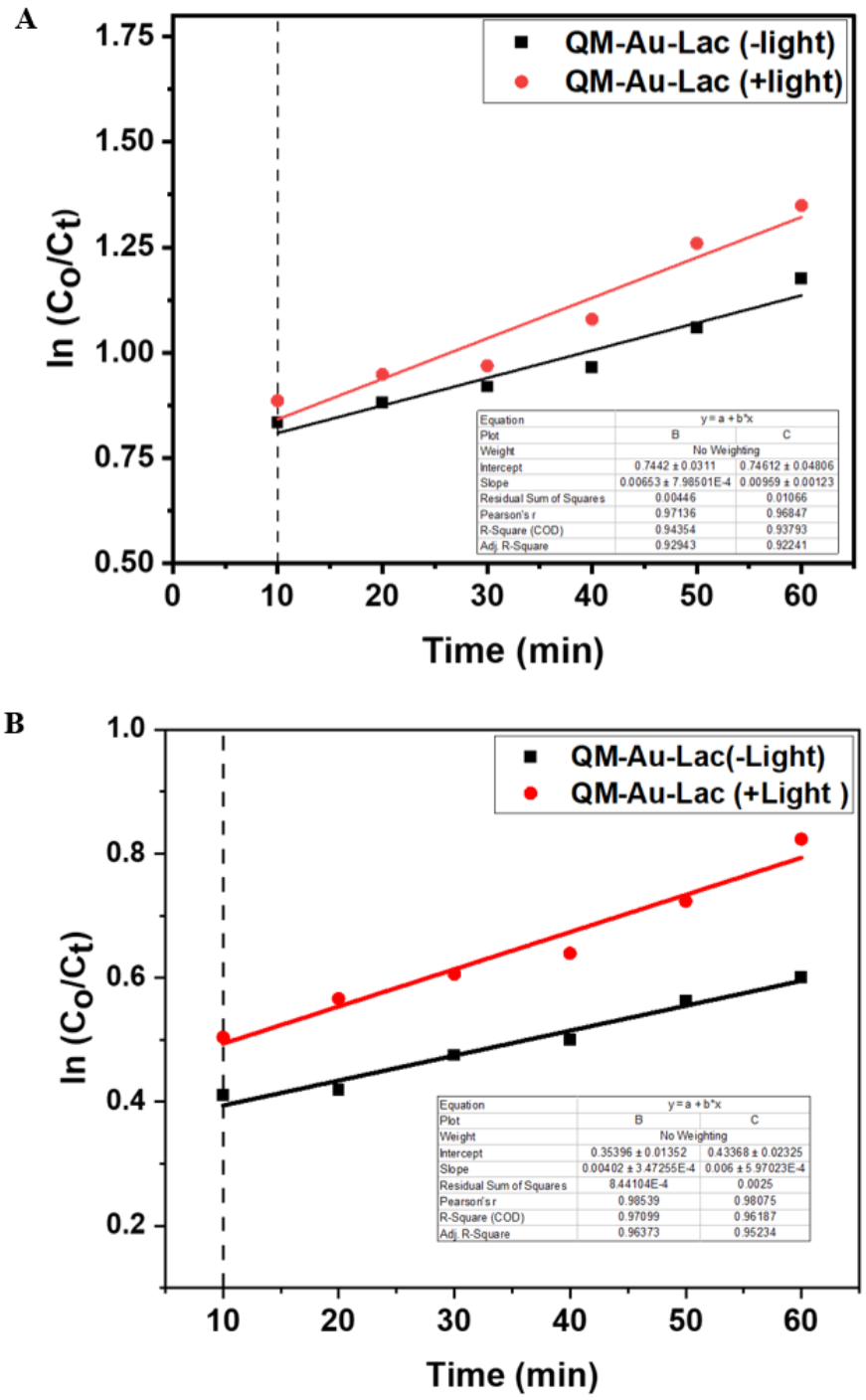

Fig. S6 The graph plotted between ln(Co/Ct) versus reaction time of (A) MB photodegradation and (B) RhB photodegradation after treating with QM-Au-Lac.

Table S3. Table representing the obtained rate constant (k) calculated from the slope of the plotted $\ln(C_o/C_t)$ versus reaction time graph in Fig. S6, along with the R^2 value.

Photodegradation using QM-Au-Lac of	Rate constant (k/min^{-1})	R^2 value
MB (-light)	0.006	0.93
MB (+light)	0.009	0.92
RhB (-light)	0.004	0.96
RhB (+light)	0.006	0.95

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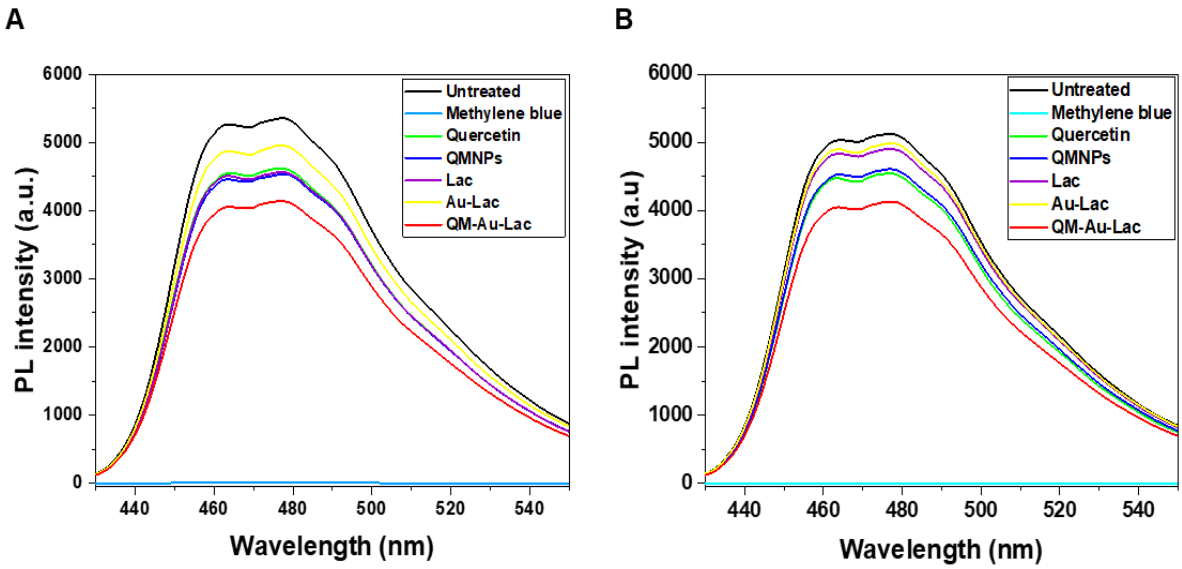

Fig. S7 Photoluminescence spectra of 1,3-diphenylisobenzofuran (DPBF) (λ_{ex} : 410 nm) treated with methylene blue (positive control), quercetin, QMNPs, Lac, Au-Lac, and QM-Au-Lac for 1 h, (A) without light and (B) with red light irradiation for 10 minutes.

Table S4 Table representing the percentage of GSH content after treatment with different treatment groups. The results have been calculated based on the graphs in Fig. 1F.

GSH content % after treating with	Without light	With light
H_2O_2 (1mM)	6	6
Quercetin	94±3.1	86±1.8
QMNPs	85±1.4	78±1
Lac	90±4	86±0.7
Au-Lac	88±3.8	88±2
QM-Au-Lac	78±0.8	72±0.4

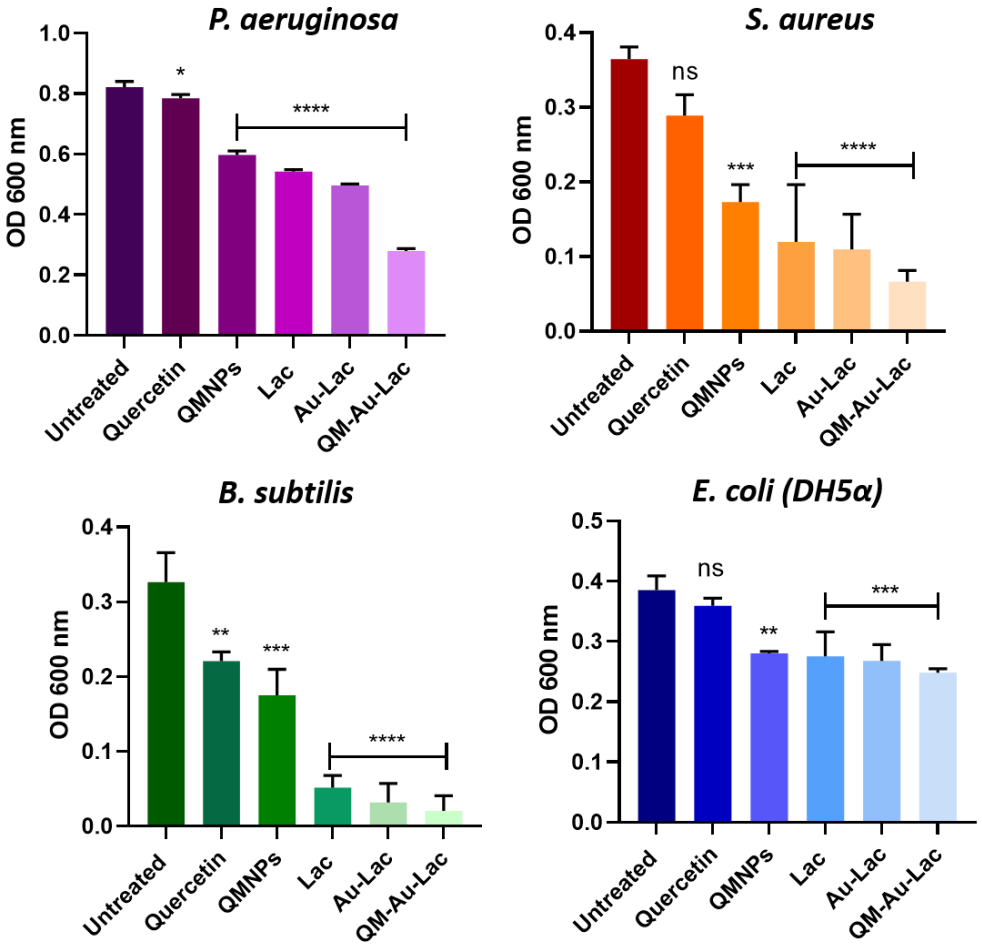

Fig. S8 Killing efficacy of quercetin, QMNPs, Lac, Au-Lac, and QM-Au-Lac against pathogenic bacteria (A) *P. aeruginosa*, (B) *S. aureus*, (C) *B.subtilis*, and (D) *E. coli* on co-incubation for 24 h.

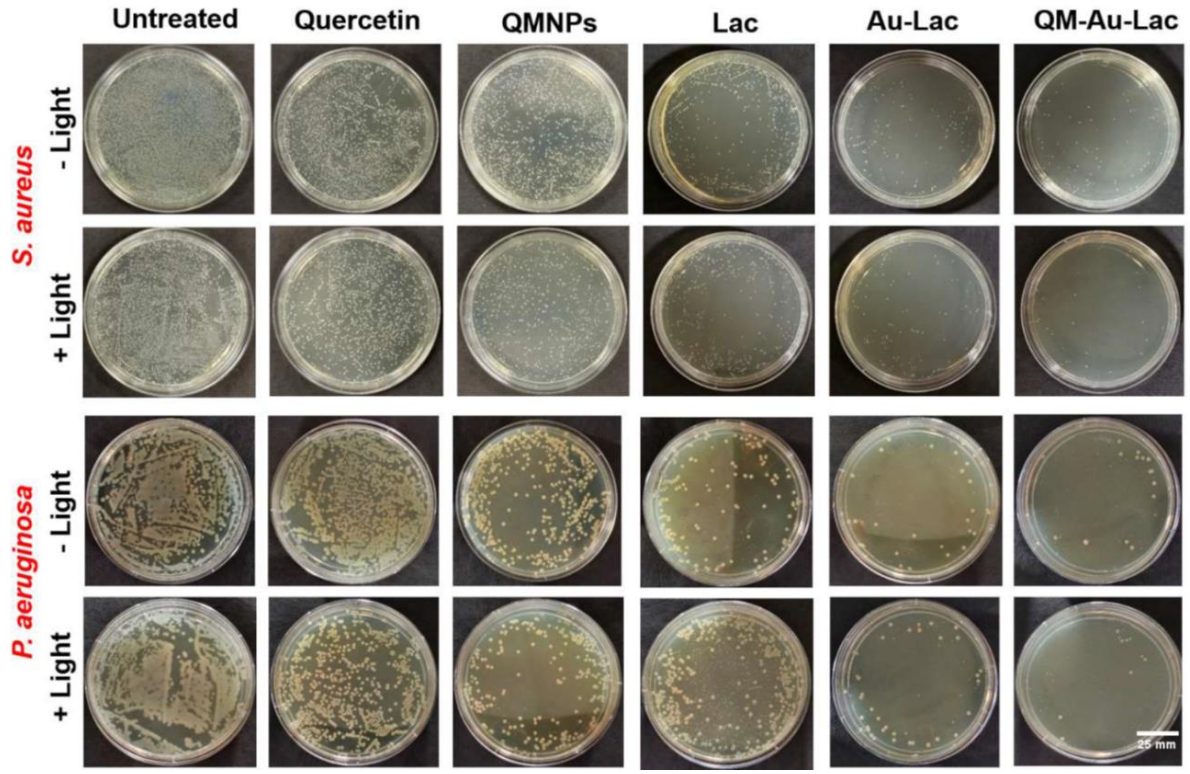

Fig. S9 Photographs of the bacterial colonies of the biofilms treated with quercetin, QMNPs, Lac, Au-Lac, and QM-Au-Lac. Scale bar is 25 mm.

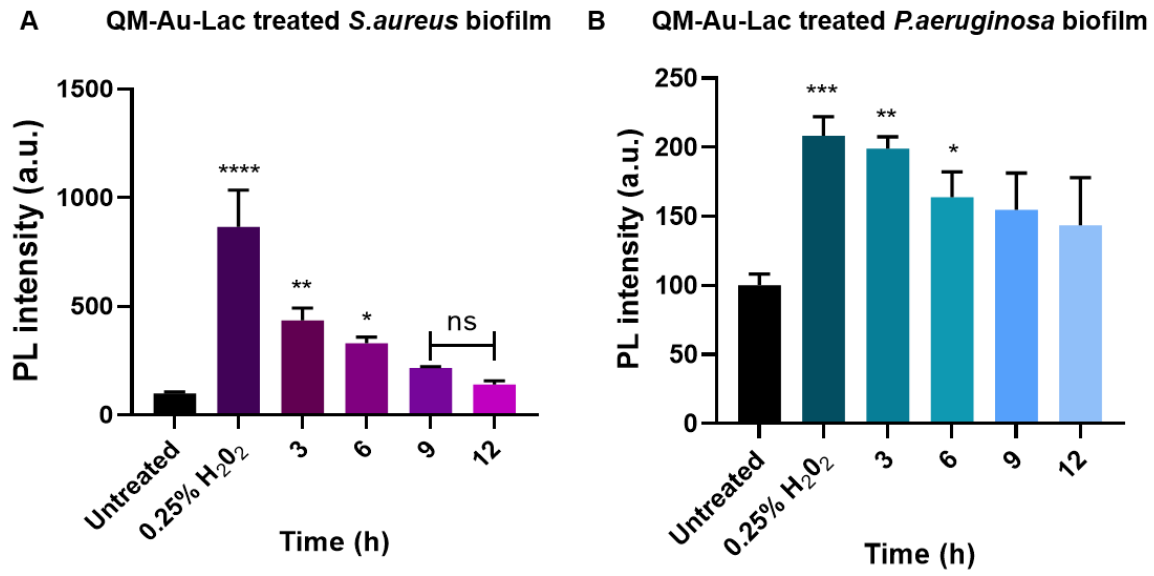

Fig. S10 Graph representing reactive oxygen species generated by QM-Au-Lac treated *S. aureus* and *P. aeruginosa* biofilms as calculated from the PL using DCFDA dye. Statistical significance is represented by ** ($p<0.01$), *** ($p < 0.001$), and **** ($p < 0.0001$). The values are represented as mean ± standard deviation of three individual experiments.

Table S5 Table representing the ROS fold change observed in *S. aureus* and *P. aeruginosa* after treatment with different treatment groups for 3 h. The results have been calculated based on the graphs in Fig. 3A.

S. aureus biofilm treated with	ROS fold change (Without light)	ROS fold change (With light)
H_2O_2 (0.25%)	2.3 ±0.1	2.7±0.07
Quercetin	1.8±0.05	1.9±0.07
QMNPs	2.0±0.02	2.0±0.03
Lac	1.7±0.0	1.7±0.03
Au-Lac	1.7±0.01	1.7±0.02
QM-Au-Lac	2.1±0.01	2.2±0.05
P. aeruginosa biofilm treated with	ROS fold change (Without light)	ROS fold change (With light)
H_2O_2 (0.25%)	2.7±0.14	2.9±0.03
Quercetin	1.5±0.05	2.0±0.09
QMNPs	1.9±0.08	2.0±0.06
Lac	1.7±0.03	1.8±0.03
Au-Lac	1.6±0.11	1.8±0.03
QM-Au-Lac	2.3±0.11	2.6±0.08

Table S6 Table representing the lipid droplet content observed in *S. aureus* and *P. aeruginosa* biofilms stained with nile red dye after treating with different treatment group for 24 h. The results have been calculated based on the graphs in Fig. 3B.

S. aureus biofilm treated with	Lipid droplet content % (Without light)	Lipid droplet content % (With light)
Quercetin	47.6±3.1	38.2±6.5
QMNPs	39.5±4.7	24.9±5.9
Lac	59.7±5.6	46.0±8.3
Au-Lac	51.3±2.1	42.1±4.1
QM-Au-Lac	27.7±4.7	10.2±5.6
P. aeruginosa biofilm treated with	Lipid droplet content % (Without light)	Lipid droplet content % (With light)
Quercetin	44.2±0.7	37.2±4.0
QMNPs	38.2±1.9	29.6±5.0
Lac	62±7.8	51.8±1.4
Au-Lac	52.2±1.6	51.2±3.1
QM-Au-Lac	28.9±3.1	17.6±7.2

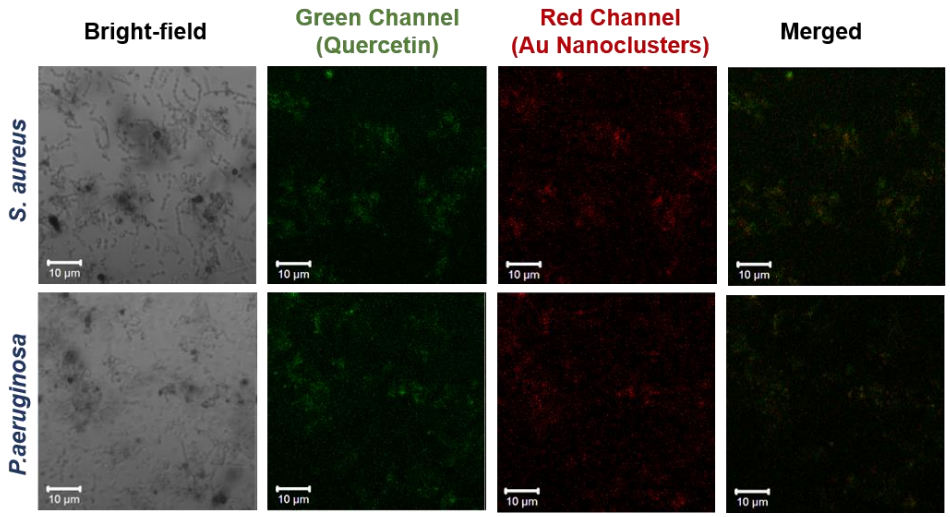

Fig. S11 CLSM images of QM-Au-Lac-treated *S. aureus* and *P. aeruginosa* biofilm recorded using 405 nm laser. Scale bar is 10 µm.

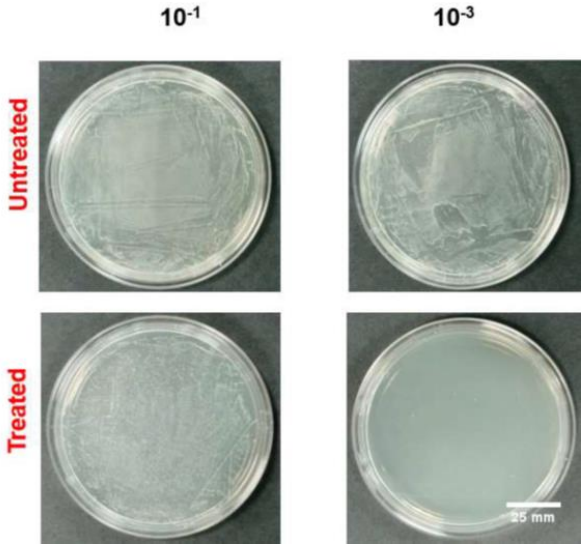

Fig. S12 Photographs of the bacterial colonies of the *P. aeruginosa* biofilm untreated (control) and QM-Au-Lac-treated. Scale bar is 25 mm.

Reference

1. Roy, S.; Debasmita, D.; Dey, U.; Ghosh, S. S.; Chattopadhyay, A., Unveiling the cytotoxic potential of quercetin-loaded magnetic bacterial bots against cervical cancer. *ACS Applied Materials & Interfaces* **2025,** *17* (4), 5799-5812.