Electronic Supplementary Material (ESI) for Chemical Communications

Retention of nisin activity at elevated pH in an organic acid complex and gold nanoparticle composite

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Electronic Supplementary Information

Materials

Nisin, potassium bromide (IR grade), 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE), propidium iodide (PI), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) and 3,3'-dipropylthiadicarbocyanine iodide ($DiSC_35$) were obtained from Sigma-Aldrich Chemicals, USA. Glutaraldehyde, trisodium citrate 2-hydrate and organic acids which included citric acid (CA), lactic acid (LA), ascorbic acid (AA), oxalic acid (OA) and gallic acid (GA) were purchased from Merck, Mumbai, India. HEPES buffer was procured from Sisco Research Laboratories (SRL), Mumbai, India.

Bacterial strains and growth conditions

The bacterial strains used in the present investigation comprised of *Micrococcus luteus* ATCC 9341 (*M. luteus*) and *Listeria monocytogenes* Scott A (*L. monocytogenes*). The growth media used for propagating the bacterial strains were purchased from HiMedia, Mumbai, India. Prior to

experiments, the bacterial strains were propagated in Brain-Heart Infusion (BHI) broth at 37^{o}C and 180 rpm for 12 h.

Preparation of nisin solution

A 10 mg/mL stock solution of nisin (equivalent to 10,000 IU/mL) was prepared in 0.75% NaCl solution (pH 5.5 adjusted with 0.1 N HCl). The stock solution was filter-sterilized using a 0.2 µm membrane filter (Pall Life Sciences, USA) and stored in -20^{o}C.

Preparation of nisin-organic acid complex

Initially a 6.8 mM stock of all the organic acids was prepared in water. To prepare nisin-organic acid complex, the respective stock solutions of nisin and various organic acids were mixed in appropriate proportions to yield a solution wherein the final acid concentration was 3.4 mM and nisin concentration was 50 IU/mL, 100 IU/mL and 200 IU/mL, respectively. The pH of the solution was adjusted to various levels as per experimental requirements with either 0.1 N HCl or 0.1 N NaOH. Subsequently the contents of the solution were allowed to mix thoroughly in a shaking incubator at room temperature (25^{o}C) for 2 h to facilitate formation of nisin-organic acid complex. The complex solutions were always prepared fresh prior to experiments unless stated otherwise.

FT-IR analysis of nisin-organic acid complex

FT-IR spectra of nisin-organic acid complex samples (corresponding to 200 IU/mL nisin concentration) were recorded in KBr pellets at 4 cm^{-1} resolution in a spectrometer (Spectrum

One, Perkin-Elmer). Five scans were performed for every sample in the range of 4000 to 450 cm^{-1}. A background spectrum for pure KBr was also measured.

Circular Dichroism (CD) spectra of nisin-citric acid complex

For CD spectra analysis, separate aliquots of nisin solution (pH 5.5 and pH 8.0, each corresponding to 200 IU/ml nisin concentration) and nisin-citric acid complex solution (pH 7.0 and pH 8.0, each corresponding to 200 IU/mL nisin concentration) were prepared in 10 mM phosphate buffer of respective pH. The CD spectra of each of these samples were recorded between 240 and 190 nm on a J-815 spectropolarimeter (JASCO) with a 1 mm path length cuvette at 25°C. CD spectra of the respective buffers were also recorded and subtracted from the sample readings to eliminate the background signal. For every sample, eight scans were recorded and averaged in order to improve the signal-to-noise ratio. Data were digitally acquired every 1 nm and the obtained spectra was smoothed using the instruments in-built program.

Preparation of nisin-gold nanoparticle composite

Synthesis of citrate-stabilized gold nanoparticles (AuNPs) was accomplished by following a standard procedure of citrate reduction of $HAuCl_4$ as described previously.[1] Following synthesis, a 1.0 mL portion of AuNP solution was taken in a cuvette and the UV-vis extinction spectrum was recorded (Cary 300, Varian, USA). An aliquot of the synthesized AuNP sample was also subjected to transmission electron microscope (TEM) analysis. The TEM images of AuNPs were subjected to ImageJ software analysis (http://rsb.info.nih.gov/ij) which revealed the average particle size as 12.0 ± 2.0 nm. For studying nisin-AuNP interaction in solution, citrate-stabilized AuNP solution (0.25 nM) was taken in a series of microcentrifuge tubes, to which varying

volumes of nisin stock solution was added drop wise to achieve adequate mixing and to attain a final nisin concentration of 25 IU/mL, 50 IU/mL, 100 IU/mL, 150 IU/mL, 200 IU/mL and 300 IU/mL. Following incubation at room temperature for 30 min, UV-vis extinction spectrum of the samples was recorded in a spectrophotometer at a wavelength set from 400-800 nm. All measurements were taken from three independent experimental samples. Aliquots of the sample from the series were also subjected to TEM analysis. The nisin-AuNP composite was prepared by interacting AuNP solution with nisin stock solution as mentioned previously so as to achieve a final nisin concentration of 50 IU/mL, 100 IU/mL and 200 IU/mL. Following incubation of nisin and AuNP solution for 30 minutes, the solution was centrifuged at 15,000 rpm for 20 min. The pellet representing nisin-AuNP composite was resuspended in sterile water and pH of the composite was adjusted separately to 5.5, 7.0 and 8.0 for further experiments. UV-vis extinction spectrum and TEM analysis of nisin-AuNP composite was pursued as described earlier.

Agar well diffusion assay

Agar well diffusion assay was conducted to ascertain the antimicrobial activity of nisin, nisin-organic acid complex solutions, nisin-AuNP composite, various organic acids and citrate-stabilized AuNP solution. As per requirement of various experiments, pH of the samples was adjusted to various levels. The assay plates had a bottom layer of BHI agar (1.5% agar), which was overlaid with BHI soft agar (0.8% agar) seeded with 10^6 cells of freshly grown target cells of *M. luteus* ATCC 9341. Requisite number of holes of 6 mm diameter was punched in all the assay plates. To each well, 30 µl of test sample was added. The plates were pre-incubated at 4°C for 3 h to facilitate diffusion of the sample followed by incubation at 37°C for 24 h. The

antimicrobial activity of the samples was determined by observing the zone of inhibition produced around the wells.

cFDA-SE leakage assay

Target cells of *M. luteus* ATCC 9341 were labeled with 5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE) as follows: Overnight grown cells of *M. luteus* ATCC 9341 were harvested by centrifugation at 8,000 rpm for 5 min. The cell pellet was washed twice with sterile phosphate buffer and labeled with cFDA-SE (final concentration of 50 μM) at 37°C for 20 min. The labeling reaction was terminated by pelleting the cells followed by washing of cells twice with phosphate buffer to remove excess cFDA-SE molecules. The labeled cells (approximately 10^6 CFU/mL) were treated with nisin, nisin-organic acid complex and nisin-AuNP composite solutions of varying concentration and pH at 37^{o}C and 180 rpm for 3 h. In case of control samples, pH adjusted buffer solutions, various organic acid solutions or citrate-stabilized AuNPs were added to labeled cells and incubated under the same conditions. Subsequently all the samples were centrifuged at 8,000 rpm for 10 min. Leakage of carboxyfluorescein from cells was determined by measuring fluorescence of the cell free supernatant at an excitation wavelength of 488 nm and emission wavelength of 518 nm in a spectrofluorimeter (FluoroMax-3, HORIBA). The fluorescence measurements were recorded after subtracting the fluorescence of effluxed dye from control samples. Fluorescence measurements were taken for three independent experimental samples. The fluorescence intensity of cFDA obtained for samples treated with nisin solution of varying concentration and pH was expressed as maximum nisin activity (100%). The fluorescence intensity of cFDA obtained for samples treated with nisin-organic acid

complex and nisin-AuNP composite solutions was compared with that obtained for nisin of corresponding concentration and pH and expressed as % nisin activity.

Determination of minimum inhibitory concentration (MIC)

Target bacterial cells of *M. luteus* ATCC 9341were inoculated at 1% level in micro titre wells having the requisite growth medium and grown overnight at 37^{o}C and 130 rpm in presence of varying concentrations of nisin, nisin-citric acid complex and nisin-AuNP composite solutions (2.5-640 IU/mL nisin concentration) adjusted to various pH (5.5, 7.0 and 8.0). Bacterial cell growth was determined by measuring absorbance at 600 nm in a micro titre plate reader (Infinite M200, TECAN, Switzerland). MIC of the antimicrobial agents was recorded as the lowest concentration which resulted in OD_{600} reading of <0.1, indicating lack of cell growth. The MIC values were calculated from three independent experiments, each having three replicas. Data analysis and calculation of standard deviation was performed with Microsoft Excel 2010 (Microsoft Corporation, USA).

Cell viability assay

Approximately 10^6 CFU/mL of target pathogens *M. luteus* ATCC 9341 and *L. monocytogenes* Scott A (in PBS) were interacted with nisin, nisin-citric acid complex and nisin-AuNP composite solutions whose pH was adjusted to various levels. The nisin concentration in the samples corresponded to 200 IU/mL. Viable cell numbers (Log_{10} CFU) were determined at regular intervals by serial dilution and plating.

PI uptake assay

A 1.5 mM stock solution of PI was prepared in sterile MilliQ water and stored at 4°C. Approximately 10^6 CFU/mL of target bacteria *M. luteus* ATCC 9341 (in PBS) was treated with nisin, nisin-citric acid complex and nisin-AuNP composite solutions of varying concentration and pH at 37°C and 180 rpm for 3 h. In case of control samples, pH adjusted buffer solutions, citric acid solution or citrate-stabilized AuNPs were added to cells and incubated under the same conditions. Subsequently, cells were washed with PBS and PI was added to both treated cells and control samples at a final concentration of 30 μM. After 30 min of incubation in a circulating water bath incubator (Amersham) set at 37°C, samples were centrifuged and washed in distilled water to remove excess dye. The cells were resuspended in PBS and fluorescence was measured in a spectrofluorimeter (FluoroMax-3, HORIBA) at an excitation wavelength of 535 nm and emission wavelength of 617 nm. Fluorescence data for each sample was normalized with the optical density at 617 nm. The values obtained for control samples were subtracted from all experimental values. Fluorescence measurements were taken for three independent samples. The fluorescence intensity of PI obtained for samples treated with nisin solution of varying concentration and pH was expressed as maximum nisin activity (100%). The fluorescence intensity of PI obtained for samples treated with nisin-citric acid complex and nisin-AuNP composite solutions was compared with that obtained for nisin of corresponding concentration and pH and expressed as % nisin activity.

Fluorescence microscopy

Approximately 10^6 CFU/mL of target bacteria *M. luteus* ATCC 9341 (in PBS) were treated with nisin, nisin-citric acid complex and nisin-AuNP composite solutions (each corresponding to 200

IU/mL nisin concentration) of varying pH (5.5 and 8.0) at 37^{o}C and 180 rpm for 3 h. In case of control sample, pH adjusted buffer was added to cells and incubated under the same conditions. Subsequently, cells were washed twice with PBS and both the treated and control cells were labeled with cFDA-SE and PI as mentioned previously. The stained cells were then fixed in 2.5% glutaraldehyde and washed twice with phosphate buffer. A 10 µl aliquot of the fixed sample was spotted on a clean glass slide, air dried and observed under fluorescence microscope (Eclipse Ti-U, Nikon) with a filter that allowed blue light excitation for cFDA-SE and green light excitation for PI stained cells. Images of the treated and control cells were recorded.

Effect of membrane potential on antimicrobial activity

M. luteus ATCC 9341 cells were pre-treated with CCCP to collapse the transmembrane proton motive force as described previously.[2] Approximately 10^7 CFU/mL CCCP treated and untreated cells (control) were suspended in PBS and incubated with nisin (200 IU/mL, pH 5.5 and 8.0), nisin-citric acid complex (corresponding to 200 IU/mL nisin concentration, pH 8.0) and nisin-AuNP composite (corresponding to 200 IU/mL nisin concentration, pH 8.0) for 3 h and 6 h at 37^{o}C. Survival of treated and control cells were compared by plating the samples and determining the viable cell count.

Membrane depolarization assay

M. luteus ATCC 9341 cells were grown till mid-logarithmic phase (OD_{600} = 0.4-0.5). The cells were harvested by centrifugation, washed with a buffer solution (5 mM HEPES buffer, 5 mM glucose, pH 7.2) and suspended in the same buffer to an OD_{600} of 0.05. The cell suspensions were incubated with 0.4 µM $DiSC_35$ for 1h at 37^{o}C followed by the addition of 100 mM KCl.

Cell suspension (1.0 mL) was placed in separate cuvettes to which nisin (200 IU/mL, pH 5.5 and 8.0), nisin-citric acid complex (corresponding to 200 IU/mL nisin concentration, pH 8.0) and nisin-AuNP composite (corresponding to 200 IU/mL nisin concentration, pH 8.0) solution was added and the fluorescence readings were monitored periodically in a spectrofluorimeter (FluoroMax-3, HORIBA) at an excitation wavelength of 622 nm and emission wavelength of 670 nm.

Storage studies

Nisin (200 IU/mL, pH 5.5), nisin-citric acid complex (corresponding to 200 IU/mL nisin concentration, pH 5.5, 7.0 and 8.0) and nisin-AuNP composite (corresponding to 200 IU/mL nisin concentration, pH 5.5, 7.0 and 8.0) solutions were stored at 4^{o}C and room temperature (25^{o}C) for a period of seven days. Samples were periodically withdrawn during storage (1, 3, 5 and 7 days) and their antimicrobial activity was tested by interacting with 10^6 CFU/mL *M. luteus* ATCC 9341 cells for 3 h at 37^{o}C and 180 rpm. Subsequently the samples were surface plated on BHI-agar plate and viable cell numbers were enumerated after overnight incubation at 37^{o}C. In case of control samples, target cells were treated with freshly prepared nisin solution (200 IU/mL, pH 5.5) and cell viability was determined as mentioned previously. The viable cell numbers obtained from treated samples were compared with control samples and the results were expressed as % activity compared to control.

Reference

1 J. Deka, A. Paul and A. Chattopadhyay, *J. Phys. Chem. C,* 2009, **113**, 6936-6947.

2 M. L. Mangoni, N. Papo, G. Mignogna, D. Andreu, Y. Shai, D. Barra and M. Simmaco, *Biochemistry*, 2003, **42**, 14023-14035.

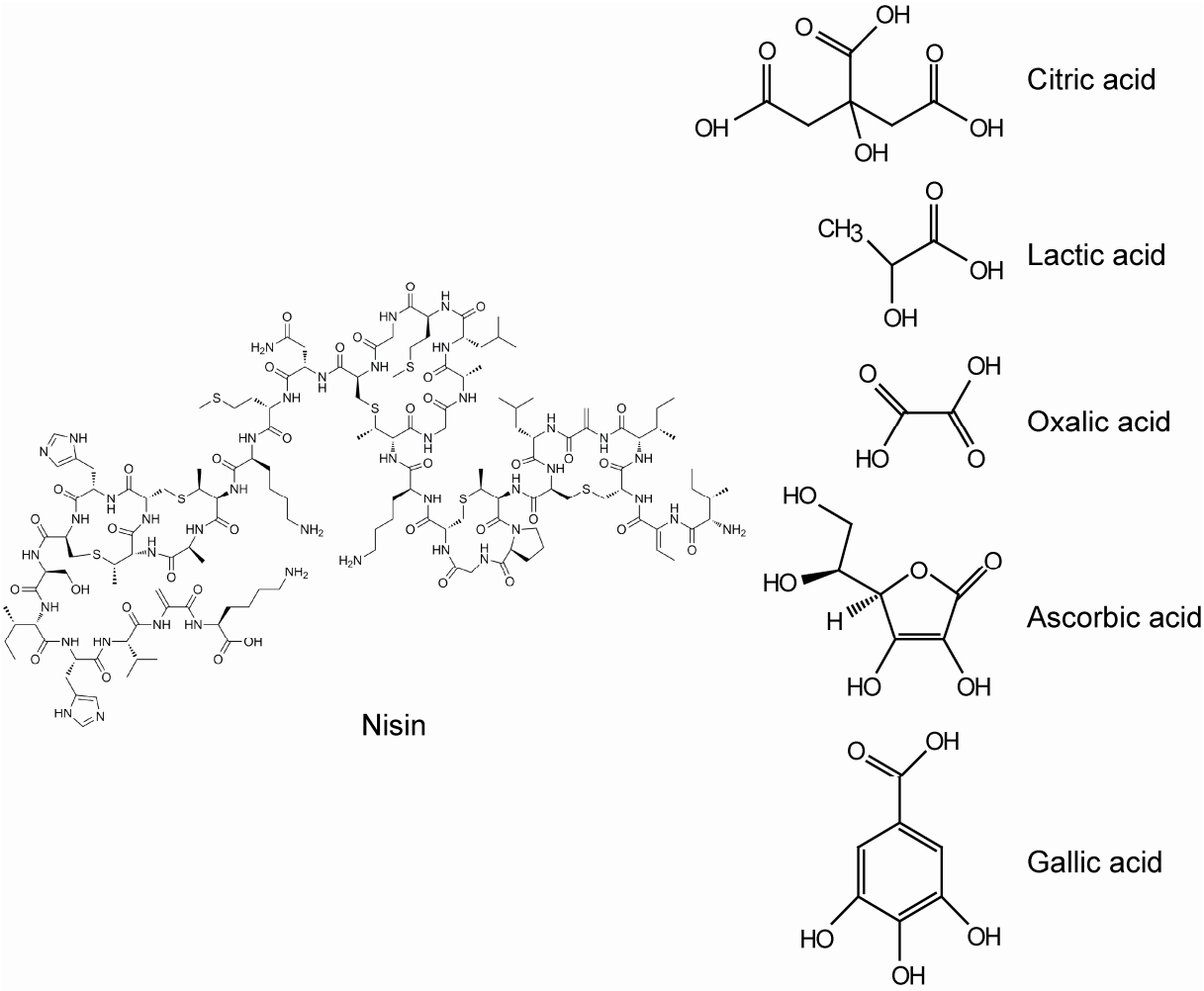

Fig. S1. Structure of nisin and various organic acids used in the study.

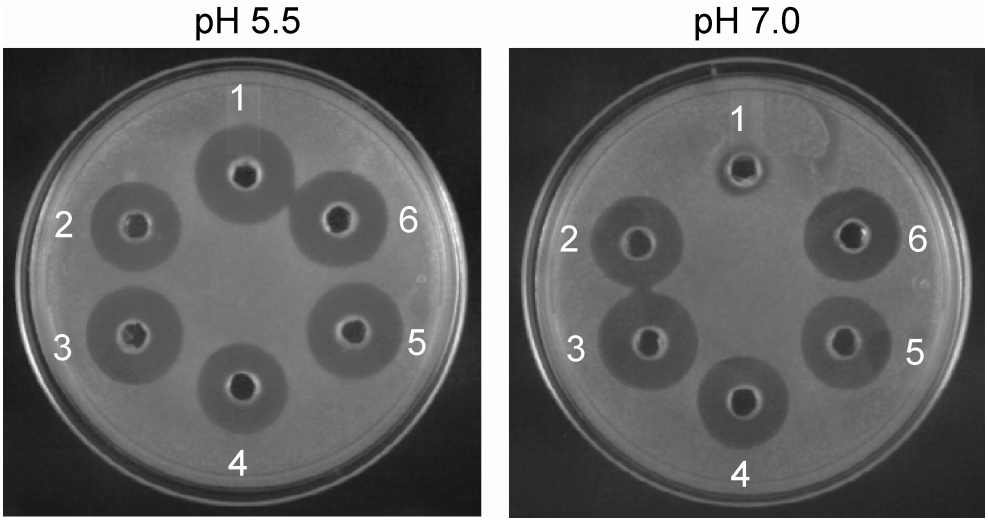

Fig. S2. Agar well diffusion assay to test the antimicrobial activity of various nisin-organic acid complex at pH 5.5 and pH 7.0 against *M. luteus* ATCC 9341. 1. Nisin, 2. Nisin-lactic acid complex, 3. Nisin-citric acid complex, 4. Nisin-gallic acid complex, 5. Nisin-ascorbic acid complex, 6. Nisin-oxalic acid complex. Nisin concentration in the samples was 200 IU/mL.

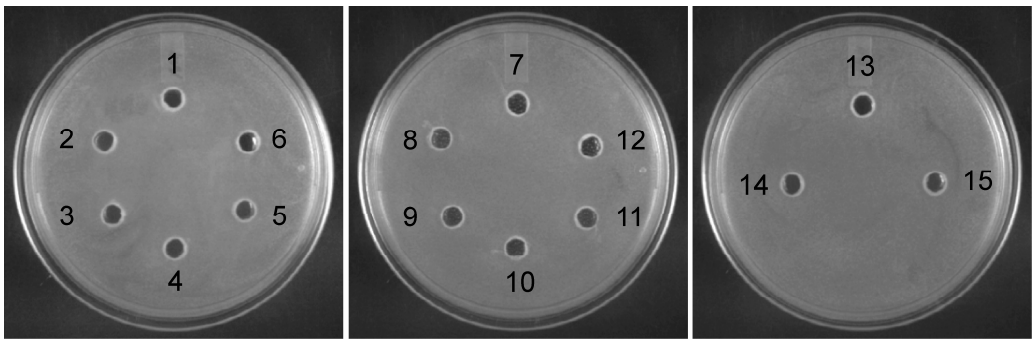

Fig. S3. Agar well diffusion assay to test the antimicrobial activity of different organic acids against *M. luteus* ATCC 9341. 1. Citric acid (pH 5.5), 2. Citric acid (pH 7.0), 3. Citric acid (pH 8.0), 4. Ascorbic acid (pH 5.5), 5. Ascorbic acid (pH 7.0), 6. Ascorbic acid (pH 8.0), 7. Oxalic acid (pH 5.5), 8. Oxalic acid (pH 7.0), 9. Oxalic acid (pH 8.0), 10. Lactic acid (pH 5.5), 11. Lactic acid (pH 7.0), 12. Lactic acid (pH 8.0), 13. Gallic acid (pH 5.5), 14. Gallic acid (pH 7.0), 15. Gallic acid (pH 8.0).

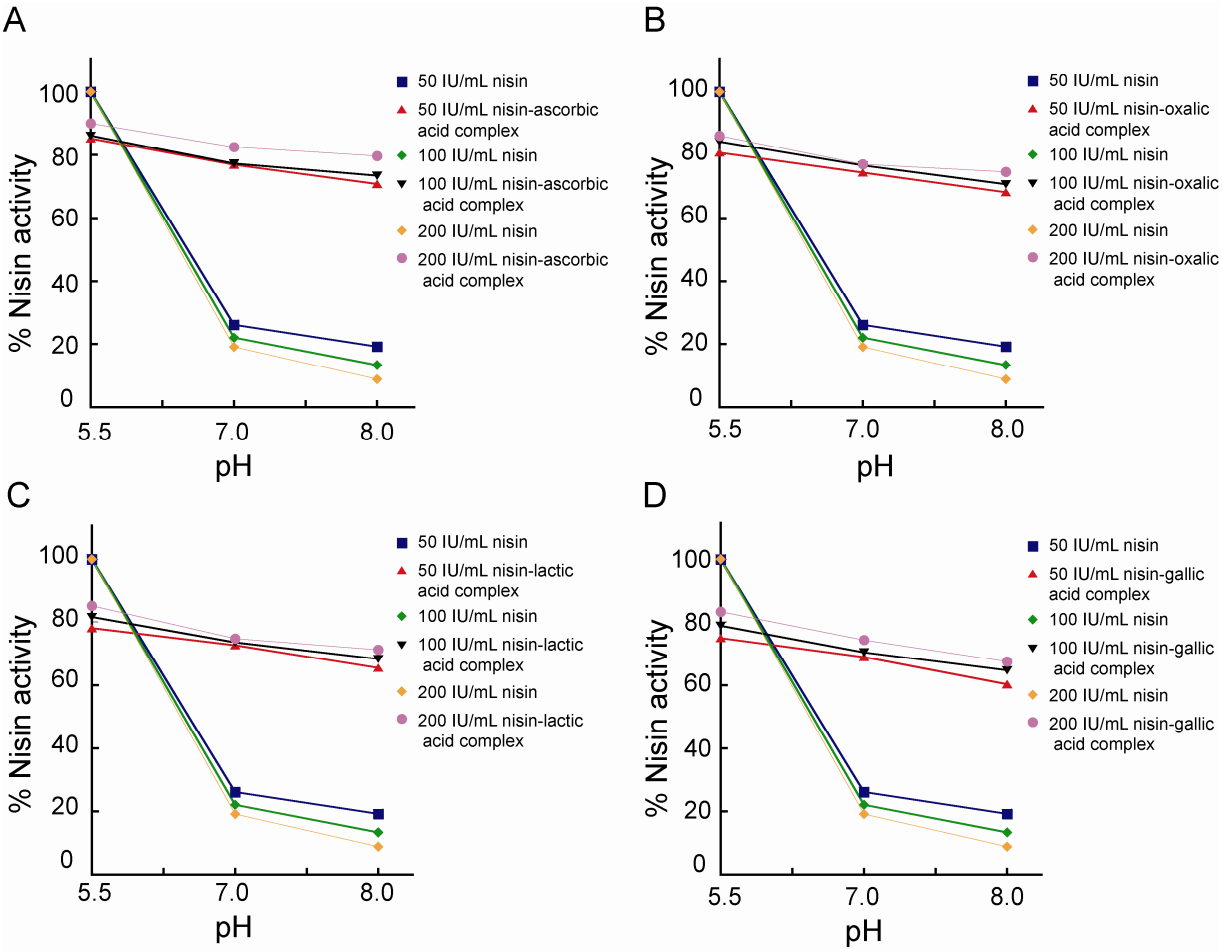

Fig. S4. cFDA-SE dye leakage assay to ascertain pore formation in *M. luteus* ATCC 9341 treated with various nisin-organic acid complex at different pH.

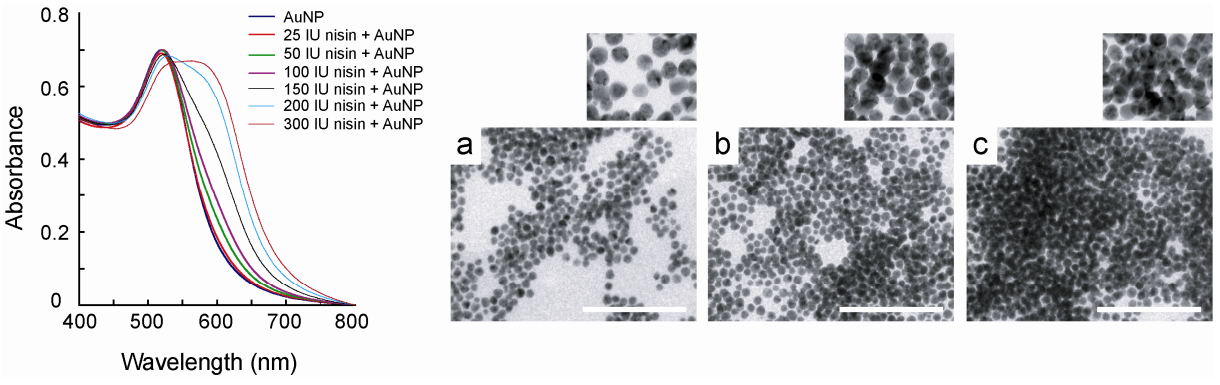

Fig. S5. UV-vis extinction spectrum of citrate-stabilized AuNPs incubated with increasing concentration of nisin is indicated in the left panel. TEM images of (a-c) AuNPs in the presence of increasing concentration of nisin (50, 100 and 200 IU) is shown in the right panel. Scale bar is 200 nm for the images.

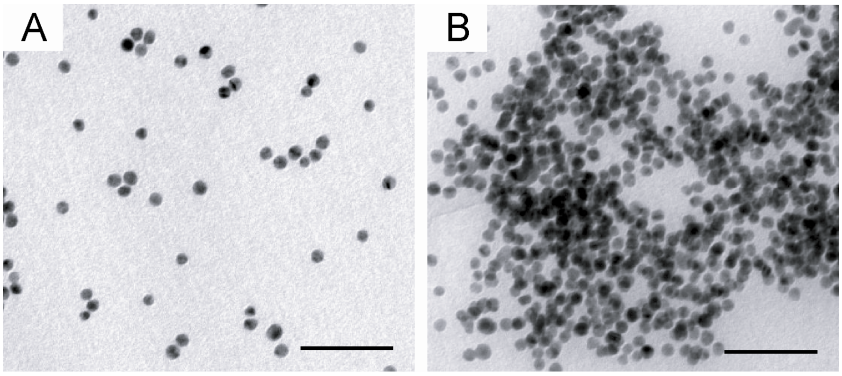

Fig. S6. TEM image of (A) as synthesized citrate-stabilized AuNPs and (B) nisin-AuNP composite (200 IU/mL nisin concentration). Scale bar for the images is 50 nm.

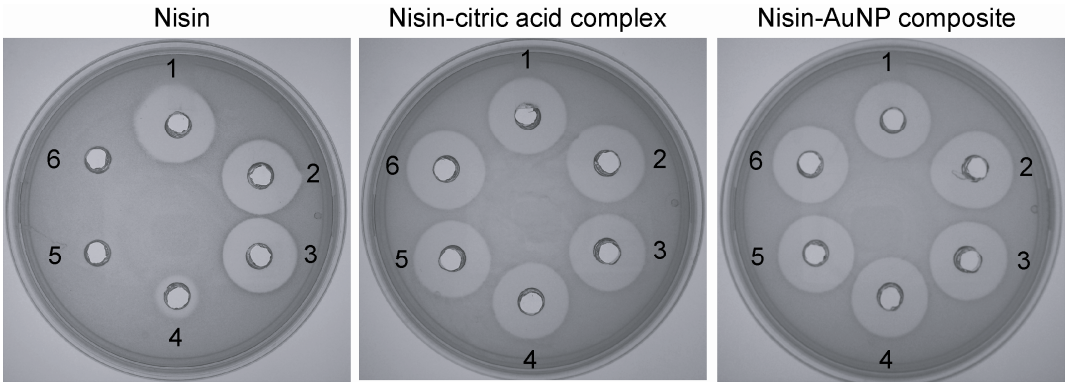

Fig. S7. Agar well diffusion assay to ascertain the activity of nisin, nisin-CA complex and nisin-AuNP composite at different pH against *M. luteus* ATCC 9341. 1. pH 3.0, 2. pH 4.0, 3. pH 5.0, 4. pH 6.0, 5. pH 7.0, 6. pH 8.0. Nisin concentration in the samples was 200 IU/mL.

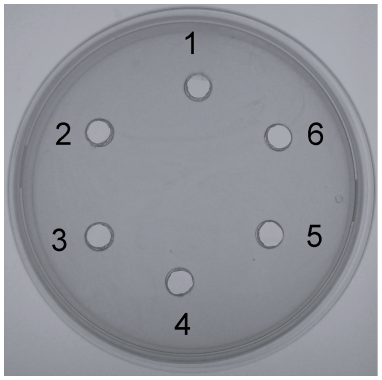

Fig. S8. Agar well diffusion assay to determine antimicrobial activity of citrate-stabilized AuNP solution of different pH against *M. luteus* ATCC 9341. 1. pH 3.0, 2. pH 4.0, 3. pH 5.0, 4. pH 6.0, 5. pH 7.0, 6. pH 8.0.

Table S1. Minimum inhibitory concentration (MIC) of nisin, nisin-citric acid complex and nisin-AuNP composite.

Sample	**MIC against *M. luteus* ATCC 9341 (IU/mL)**
Nisin (pH 5.5)	60
Nisin-CA complex (pH 5.5)	60
Nisin-CA complex (pH 7.0)	80
Nisin-CA complex (pH 8.0)	80
Nisin-AuNP composite (pH 5.5)	80
Nisin-AuNP composite (pH 7.0)	80
Nisin-AuNP composite (pH 8.0)	80

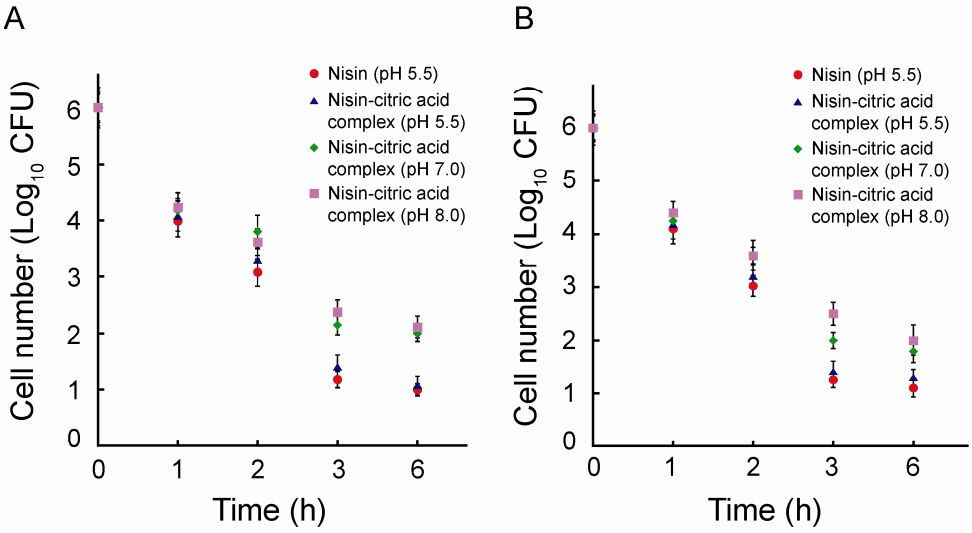

Fig. S9. Effect of nisin-citric acid complex on the viability of **(A)** *M. luteus* ATCC 9341 and **(B)** *L. monocytogenes* Scott A at various pH.

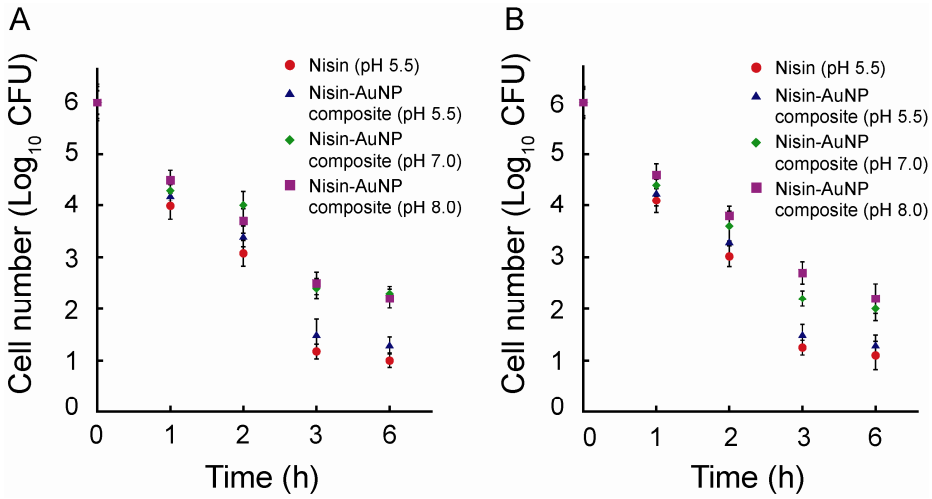

Fig. S10. Effect of nisin-AuNP composite on the viability of **(A)** *M. luteus* ATCC 9341 and **(B)** *L. monocytogenes* Scott A at various pH.

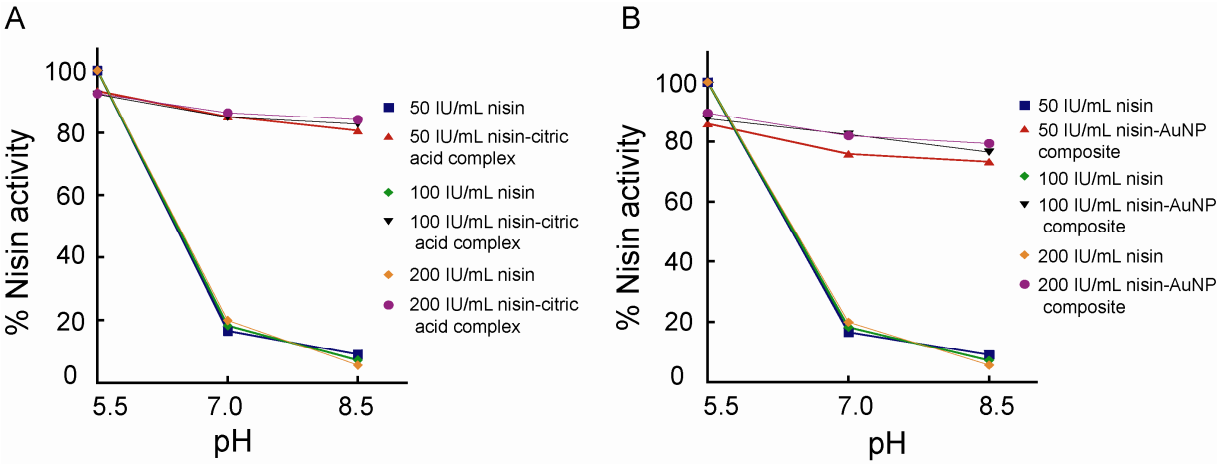

Fig. S11. PI uptake assay to study the effect of nisin-citric acid complex and nisin-AuNP composite at different pH on *M. luteus* ATCC 9341.

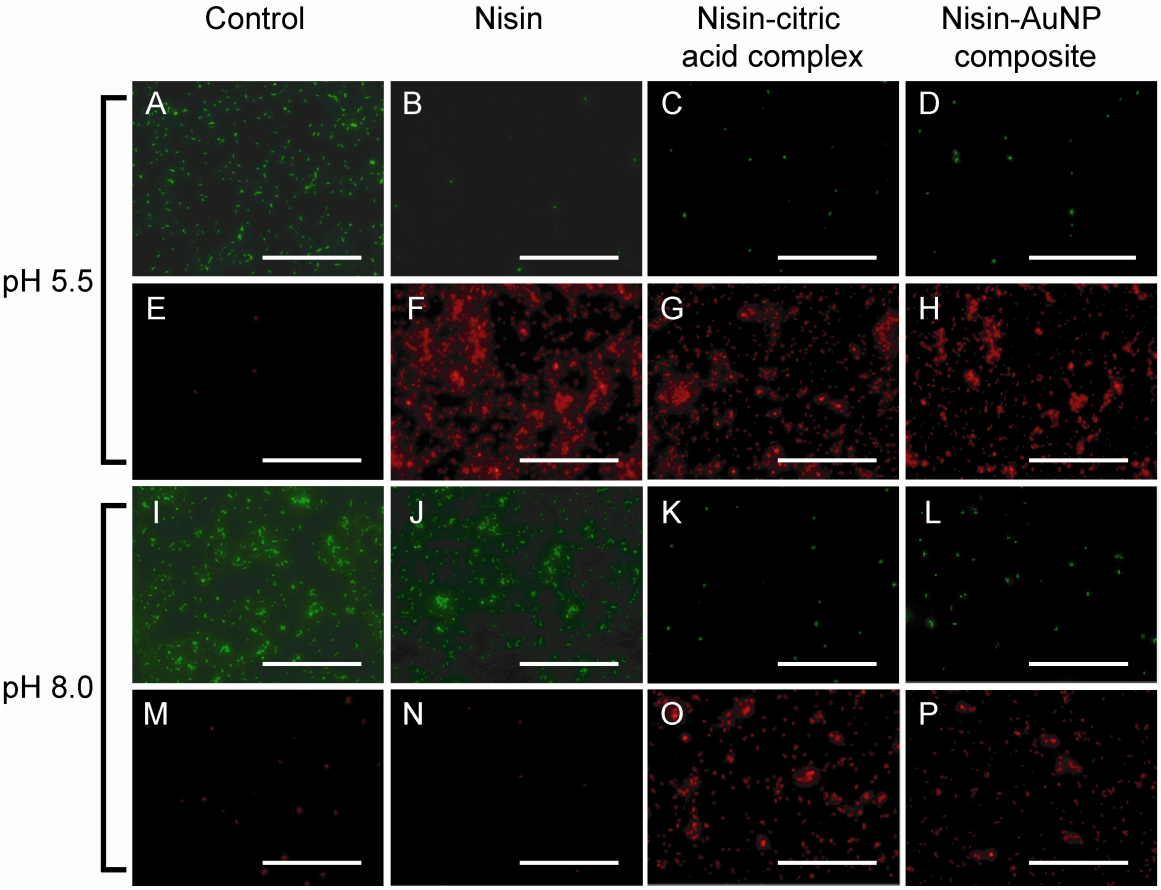

Fig. S12. Fluorescence microscopic images of *M. luteus* ATCC 9341 cells treated with nisin, nisin-citric acid complex and nisin-AuNP composite at various pH. Green cells indicate viable cells labeled with cFDA-SE **(Panel A-D and I-L)** and red cells indicate membrane damaged cells labeled with PI **(Panel E-H and M-P)**. Control samples indicate untreated bacterial cells (negative control). Scale bar for all the images is 50 µm.

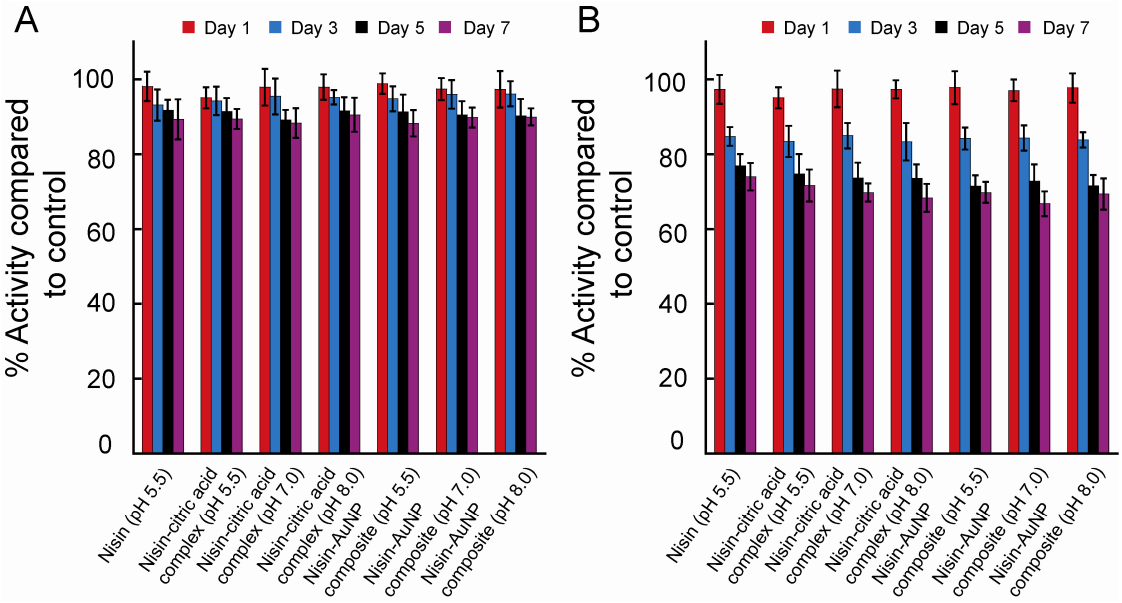

Fig. S13. Antimicrobial activity of nisin, nisin-citric acid complex and nisin-AuNP composite stored at **(A)** 4°C and **(B)** room temperature (25°C).