Supporting Information

A Biocompatible Nanocomposite Tailored to Endure the Gastric Niche Renders Effective *In vitro* Elimination of Intestinal Pathogenic Bacteria and Supports Adhesion by Beneficial Bacteria

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EXPERIMENTAL SECTION

Growth Media and Chemicals

5 (and 6)-carboxyfluorescein diacetate succinimidyl ester (cFDA-SE), pepsin, human serum albumin (HSA), 3,3'-dipropylthiadicarbocyanine iodide (DiSC35), 1.0 kDa cut-off dialysis bag, 12 KDa cut-off dialysis bag, 5-carboxy-tetramethylrhodamine N-succinimidyl ester (TAMRA-SE), 2-(4-amidinophenyl)-6-indolecarbamidine dihydrochloride (DAPI), Triton X-100, pepsin, Dulbecco's modified Eagle's Medium (DMEM), penicillin-streptomycin and MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) reagent were obtained from Sigma-Aldrich Chemicals, USA. Dimethyl sulfoxide (DMSO), glutaraldehyde and methanol were purchased from Merck, India. HEPES buffer was procured from Sisco Research Laboratories (SRL), Mumbai, India. Brain-Heart infusion (BHI) broth, deMan, Rogosa and Sharpe (MRS) broth and trichloroacetic acid (TCA) were purchased from Himedia, Mumbai, India. Fetal bovine serum (FBS) was procured from Gibco, India.

Pediocin Activity in Ped-MPE Incubated in SGF

Pediocin activity in Ped-MPE samples was ascertained by an agar well diffusion assay and cFDA-SE leakage assay as described below:

Agar Well Diffusion Assay

The assay plates had a bottom layer of BHI agar (1.5 % agar), which was overlaid with BHI soft agar (0.8% agar) seeded separately with 10^6 cells of freshly grown target cells of *L. monocytogenes* Scott A. Requisite number of holes of 6.0 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 bactericidal activity of the samples was determined by observing the zone of inhibition produced around the wells.

Fluorescence-based cFDA-SE Leakage Assay

Initially, the target cells of *L. monocytogenes* Scott A were labelled with cFDA-SE as described previously. [1] Subsequently, the labelled target cells (approximately 10^6 CFU/mL) were incubated with either Ped or Ped-MPE, which had been subjected to *in vitro* digestion in SGF (pH 2.0) or incubated in 10 mM phosphate buffer (pH 7.0) and the cells were incubated at 37^{o}C and 180 rpm for 3 h. Subsequently, all the samples were centrifuged at 6000 × g 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-4, HORIBA, Japan). The fluorescence measurements were recorded after subtracting the fluorescence of effluxed dye from control samples. The fluorescence intensity (F_T) of leaked cFDA obtained for cells treated with pure pediocin (200 AU/mL) incubated in 10 mM phosphate buffer (pH 7.0) was considered as maximum pediocin activity (100%). The fluorescence intensity (F_A) of cFDA obtained for cells treated with either Ped or Ped-MPE were then compared with that obtained for pediocin incubated in 10 mM phosphate buffer (pH 7.0) for ascertaining the relative pediocin activity. Pediocin activity was calculated as follows: $[(F_A/F_T) \times 100]$. Fluorescence measurements were taken for three independent experimental samples having three replicates each.

Characterization of MPE-SGF Interaction

Isothermal Calorimetry (ITC)

ITC measurements were made at 37°C using a VP-ITC device (MicroCal, Northampton, USA). To prevent the formation of air bubbles, the buffers were degassed under vacuum prior to experiment. MPE and pepsin were dissolved in 0.5% NaCl sterile solution pH 2.0 so as to minimize heats of dilution. To study the binding of pepsin and MPE, pepsin (0.3%) was dissolved in 0.5% NaCl solution pH 2.0 and loaded onto the cell and titrated against MPE (0.5 mg/mL). Integrated heat effects, after correction for heats of dilution, were analyzed by non-linear regression using a single site-binding model (Microcal Origin, version 5.0).

Enzyme Kinetics

In separate sets, solution of either Ped or Ped-MPE (pH adjusted to 2.0) having pediocin concentration from 10 µg/mL to 80 µg/mL were added in equal volumes to double-strength SGF (2X SGF). Subsequently, 0.5 mL of either Ped in SGF or Ped-MPE in SGF was aspirated at regular intervals, mixed with 0.5 mL TCA (10% w/v) and incubated at 37 °C for 15 min. Following incubation, the solution was centrifuged at 9,000 x g for 20 min and the absorbance of the supernatant was measured at 280 nm. All the experiments were performed in triplicates and the mean and standard deviations was calculated.

Characterization of MNP and Ped-MNC

Field Emission Transmission Electron Microscope (FETEM) Analysis

Initially, aliquots of MNP (2.0 mg/mL in sterile MilliQ water, pH adjusted to 8.2) and Ped-MNC (2.0 mg/mL MNP loaded with 800 AU/mL pediocin in sterile MilliQ water, pH

adjusted to 8.2) were prepared. Subsequently, the aqueous solutions were diluted tenfold, spotted onto carbon coated Cu-TEM grid (Pacific Grid, USA) and air dried in a laminar hood. The Cu grid was then loaded onto a specimen holder and microscopic images were captured in a FETEM microscope (JEOL 2100F, Japan) operating at 200 kV. The particle size of MNP and Ped-MNC was determined using ImageJ software (http://rsb.info.nih.gov/ij).

Dynamic Light Scattering (DLS) Analysis of MNP and Ped-MNC

For particle size estimation, a sample of MNP (2.0 mg/mL in sterile MilliQ water, pH adjusted to 8.2) or Ped-MNC (2.0 mg/mL MNP loaded with a final concentration of 800 AU/mL Ped) was dispersed in 1.0 mL sterile MilliQ water (pH adjusted to 8.2). A 0.2 mL aliquot of this solution was further diluted to 1.0 mL in sterile MilliQ water (pH adjusted to 8.2) and subjected to particle size estimation by DLS. The DLS experiments were performed in three independent sets and every set consisted of three replicates.

Thermogravimetric Analysis (TGA)

MNP and Ped-MNC (lyophilized powder ~ 8.0 mg each) were subjected to TGA (Libra TG 209, NETZSCH, Germany). The temperature range was fixed from 20 °C to 1200 °C with a heating rate of 10 °C per minute under an inert atmosphere. The raw data was subjected to fitting using the instruments built-in program.

Estimation of Loading Capacity (LC) and Encapsulation Efficiency (EE)

For estimation of LC and EE, MNPs (2.0 mg/mL in sterile 10 mM phosphate buffer, pH 7.4) were interacted with varying concentrations of Ped (4.8 µg/mL to 97.93 µg/mL) for

18 h on a rocker at 37 °C. Following incubation, the solution was centrifuged at 10,000 × g for 5 min. The pellet, which essentially represents Ped-MNC, was resuspended in sterile 10 mM phosphate buffer (pH 7.4). The level of free Ped in the supernatant (in AU/mL) was determined using a standard curve obtained from the cFDA-SE leakage assay by following the method described previously. [1] The loading capacity (LC) and encapsulation efficiency (EE) of MNP was calculated as follows:

$$LC = \frac{W_{\text{Total Ped}} - W_{\text{Free Ped}}}{W_{np}} \times 100\ \%$$

$$EE = \frac{W_{\text{Total Ped}} - W_{\text{Free Ped}}}{W_{\text{Total Ped}}} \times 100\ \%$$

where $W_{\text{Total Ped}}$ is the concentration of Ped (in mg/mL) used initially for preparing Ped-MNC, $W_{\text{Free Ped}}$ is the concentration of free Ped (in mg/mL) recovered after formation of Ped-MNC and W_{np} is the weight of MNP (in mg).

In Vitro Release Kinetics of Pediocin from Ped-MNC

To study the *in vitro* release kinetics of pediocin, Ped-MNC loaded with 800 AU/mL of pediocin was dispersed in separate sets in either 1.0 mL of 10 mM HEPES buffer (pH 7.4), 100 mM citrate buffer (pH 3.0) or sterile simulated colonic fluid (SCF), respectively. SCF was prepared as described previously. [2] The samples were incubated in an orbital shaker at 180 rpm at 37 °C. At specific time intervals (3 h, 6 h, 9 h, 12 h and 24 h) the samples were withdrawn and centrifuged at 10,000 × g for 15 min. The supernatant from various samples were transferred into a fresh microcentrifuge tube, and pediocin activity was measured based on cFDA-based dye

leakage assay as mentioned previously. The pediocin released from Ped-MNC at specific time periods was expressed as % cumulative release.

Characterization of MNP-SGF Interaction

Isothermal Calorimetry (ITC)

To study the binding of pepsin and MNP, pepsin (0.3%) was dissolved in 0.5% NaCl solution pH 2.0 and loaded onto the cell and titrated against MNP (0.5 mg/mL). The parameters of the MNP-pepsin interaction was kept same as mentioned previously for the ITC titration between MPE and pepsin.

DLS Analysis

Preparation of human serum albumin nanoparticles (HNPs) was accomplished following the standard desolvation method. [3] Subsequently, in separate sets, MNP (2.0 mg) and HNP (2.0 mg) were incubated in 1.0 mL SGF at 37 °C. At different time intervals, the solutions were centrifuged at 10,000 × g for 10 min, and the pellet was resuspended in sterile MilliQ water (pH adjusted to 8.2), diluted tenfold in the same and subjected to DLS analysis (Zeta Sizer, Malvern, UK). The DLS experiments were performed in three independent sets and every set consisted of three replicates.

Enzyme Kinetics

In separate sets, solution of either Ped (pH adjusted to 2.0) or Ped along with MNP (1.0 mg/mL and pH adjusted to 2.0) having pediocin concentration from 10 µg/mL to 80 µg/mL were added to SGF. Subsequently, 0.5 mL of either solution was aspirated at regular

intervals, mixed with 0.5 mL TCA (10% w/v) and incubated at 37 °C for 15 min. Following incubation, the solution was centrifuged at 9,000 × g for 20 min and the absorbance of the supernatant was measured at 280 nm. All the experiments were performed in triplicates and the mean and standard deviations was calculated.

Cytotoxic Assay for Pediocin, MNP and Ped-MNC

The cytotoxic effect of purified pediocin, Ped-MNC and MNPs was tested on human colon adenocarcinoma (HT-29) cell line and human embryonic kidney (HEK 293) cell line by an MTT assay following the manufacturer instruction (Sigma-Aldrich, MO, USA). HT-29 cells and HEK 293 cells were initially propagated in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (FCS), penicillin (100 µg/mL) and streptomycin (100 µg/mL) at 37 °C in a humidified atmosphere of 5.0 % CO_2. The cells were subsequently seeded in 96 well plates (10^4 cells/well) and purified pediocin (10-80 µg/mL), Ped-MNC (corresponding to a loaded pediocin concentration of 10-80 µg/mL and MNP concentration of 2.0 mg/mL) and MNPs (2.0 mg/mL) made in DMEM were added to the cells in separate sets and incubated for 24 hours under 5.0 % CO_2 at 37 °C. Following incubation, the media was aspirated and fresh DMEM containing MTT solution was added to the wells and incubated for 4 h at 37^{o}C. Subsequently, the cell viability (%) was assessed by measuring the absorbance as described previously. [4] MTT assay was performed in six independent sets and each set consisted of three replicates. Data analysis and determination of standard deviation was performed with Microsoft Excel 2010 (Microsoft Corporation, USA).

Estimation of Adhesion Process Parameters

TAMRA-SE labelled *E. faecalis* MTCC 439 or *S. aureus* MTCC 740 cells (cell numbers ranging from 4.0-8.0 $\log_{10}$ CFU per well) were allowed to adhere on HT-29 cells for 1 h. Following incubation, the following test samples were added in separate wells: (1) cFDA-SE labelled *L. plantarum* DF9 or *L. rhamnosus* GG cells (7.0 $\log_{10}$ CFU added in 500 µL sterile DMEM per well), (2) Ped-MNC (loaded with 800 AU/mL pediocin and added in 500 µL sterile DMEM per well) or pediocin (800 AU/mL pediocin and added in 500 µL sterile DMEM per well) (3) a combination of cFDA-SE labelled *L. plantarum* DF9 or *L. rhamnosus* GG cells (7.0 $\log_{10}$ CFU added in 250 µL sterile DMEM per well) and Ped-MNC (loaded with 800 AU/mL pediocin and added in 250 µL sterile DMEM per well) or pediocin (800 AU/mL pediocin and added in 500 µL sterile DMEM per well). Following 1 h of incubation, the supernatant was aspirated and HT-29 cells containing the adhered bacterial cells were treated with 0.05 % Triton X-100 to selectively lyse the mammalian cells. The suspension, was subjected to flow cytometry (FCM) and the adhesion process parameters, dissociation constant (k_d) and maximum number of adhered cells (e_m) were estimated as described previously. [5] Each experiment was performed in triplicate and the result was represented as mean ± standard deviation.

***In Vitro* Imaging Studies**

In the adhesion inhibition experiments, HT-29 cells were initially labelled with DAPI. Subsequently, HT-29 cells were incubated with TAMRA-labelled *E. faecalis* MTCC 439 (8.0 $\log_{10}$ CFU/mL) for 1 h and the adhered cells of *E. faecalis* MTCC 439 were incubated with Ped-MNC (loaded with pediocin levels of 800 AU/mL) and cFDA-SE labelled *L. plantarum* DF9 (7.0

$\log_{10}$ CFU/mL) for 1 h. Subsequently, the cells were washed with sterile PBS and the images of the adhered cells was captured using appropriate filters. [5]

References

1. Mukherjee, S.; Singh, A.K.; Adhikari, M. D.; Ramesh, A. Quantitative appraisal of the probiotic attributes and in vitro adhesion potential of anti-listerial bacteriocin-producing lactic acid bacteria. *Probiotics and Antimicrobial Proteins* **2013**, *5*, 99-109.

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RESULTS

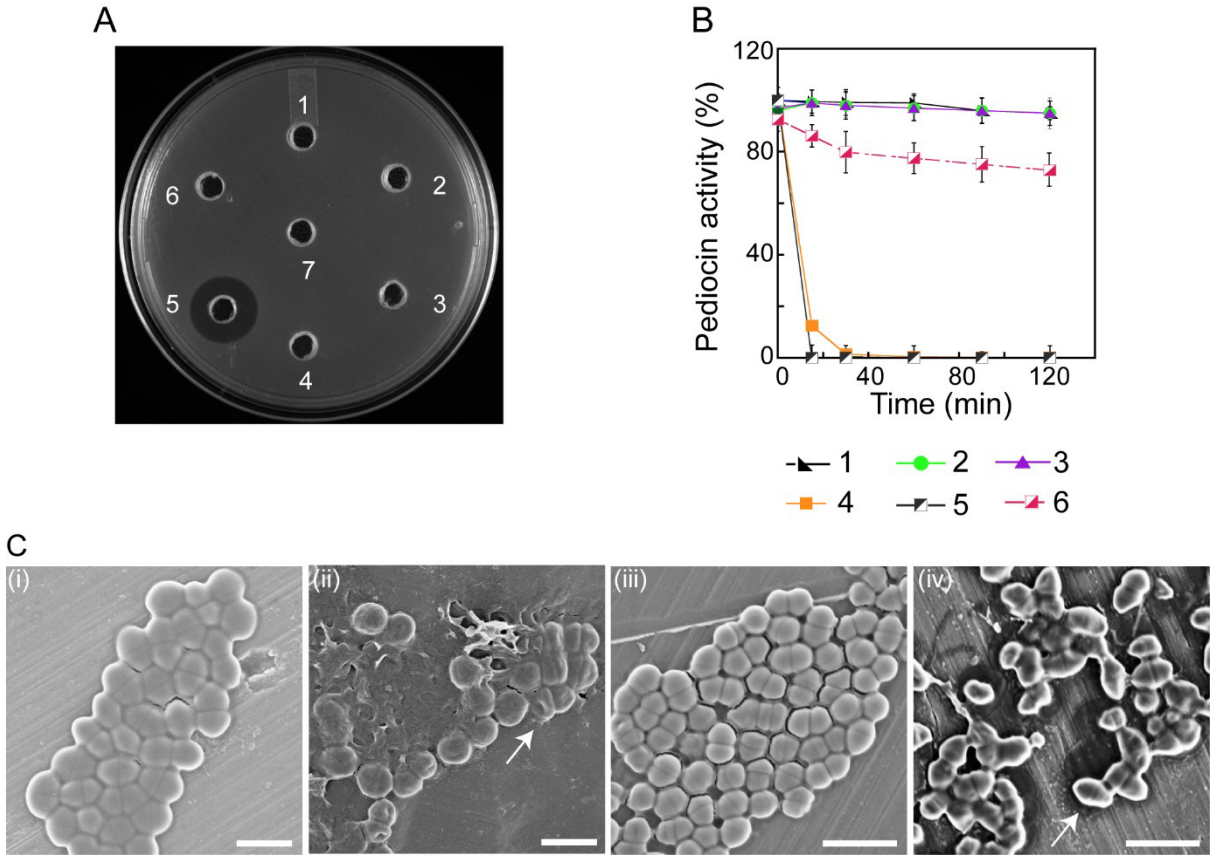

Figure S1. (A) Agar well diffusion assay performed on *L. monocytogenes* Scott A to ascertain pediocin activity in the following samples. 1. SGF, 2. HSA in SGF, 3. Pediocin-HSA complex in SGF, 4. Pediocin in SGF, 5. Pediocin-MPE complex in SGF, 6. MPE in SGF, 7. MPE in PBS. (B) cFDA-SE based dye leakage assay to ascertain pediocin activity in the following samples. 1. Pediocin (10 mM PBS), 2. Pediocin-HSA complex (10 mM PBS), 3. Pediocin-MPE complex (10 mM PBS), 4. Pediocin (SGF), 5. Pediocin-HSA complex (SGF), 6. Pediocin-MPE complex (SGF). (C) FESEM analysis to ascertain activity of pediocin-MPE complex on *S. aureus* MTCC 96 cells. (i) control cells (untreated), (ii) cells treated with pediocin, (iii) cells treated with pediocin incubated in SGF and (iv) cells treated with Pediocin-MPE complex (previously incubated in SGF). Arrows in panels (ii) and (iv) indicate damaged cells. Scale bar for the images is 2.0 µm.

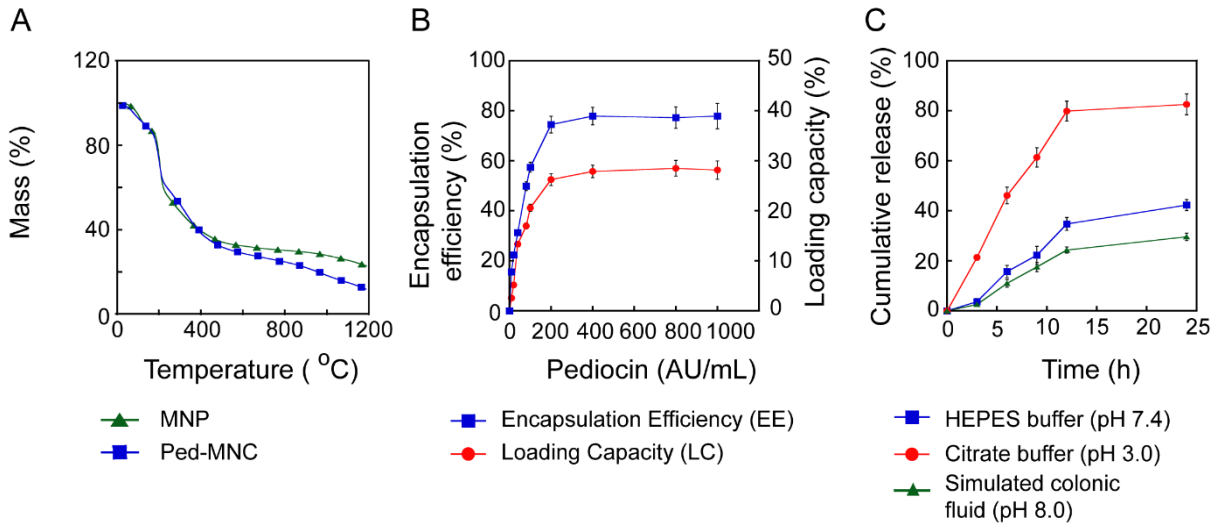

Figure S2. (A) TGA analysis of MNP and Ped-MNC. (B) Loading capacity (LC) and encapsulation efficiency (EE) of pediocin in MNP. (C) *In vitro* release kinetics of pediocin from Ped-MNC incubated in 10 mM HEPES buffer (pH 7.4), 100 mM citrate buffer (pH 3.0) and simulated colonic fluid (pH 8.0).

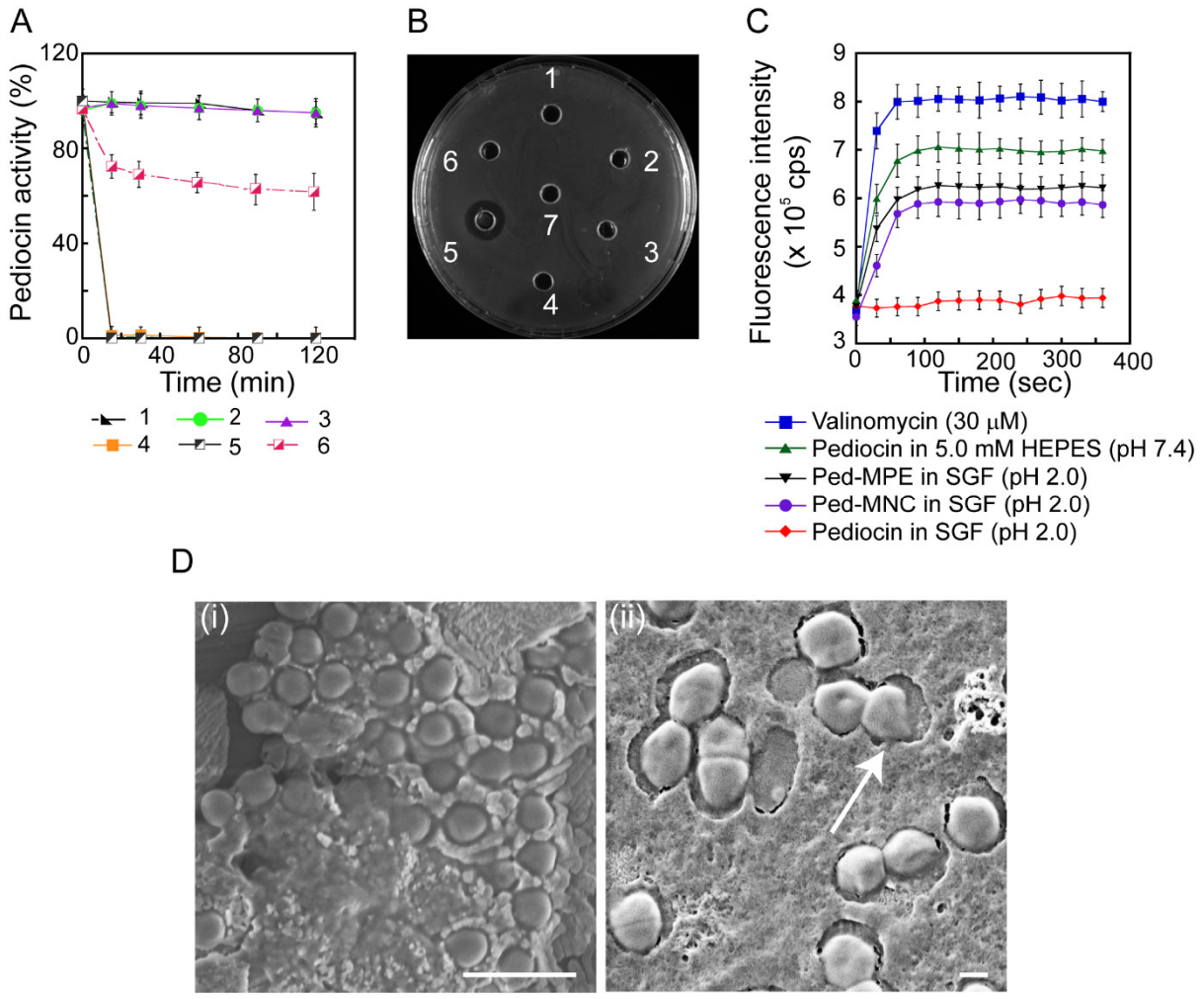

Figure S3. (A) cFDA-SE-based pediocin activity assay. 1. Pediocin (in 10 mM PBS), 2. Ped-HNC (in 10 mM PBS), 3. Ped-MNC (in 10 mM PBS), 4. Pediocin (in SGF), 5. Ped-HNC (in SGF), 6. Ped-MNC (in SGF). (B) Agar well diffusion assay performed on *L. monocytogenes* Scott A to ascertain pediocin activity. 1. SGF, 2. HNP in SGF, 3. Ped-HNC in SGF, 4. Pediocin in SGF, 5. Ped-MNC in SGF, 6. MNP in SGF, 7. MNP in PBS. (C) $DiSC_35$-based membrane potential assay to ascertain activity of Ped-MPE and Ped-MNC against *L. monocytogenes* Scott A. Valinomycin (30 µM) was used as a positive control. (D) FESEM analysis of *S. aureus* MTCC 96 treated with (i) MNP and (ii) Ped-MNC (previously incubated in SGF). Arrow in panel (ii) indicates damaged cell. Scale bar for the images corresponds to 1.0 µm.

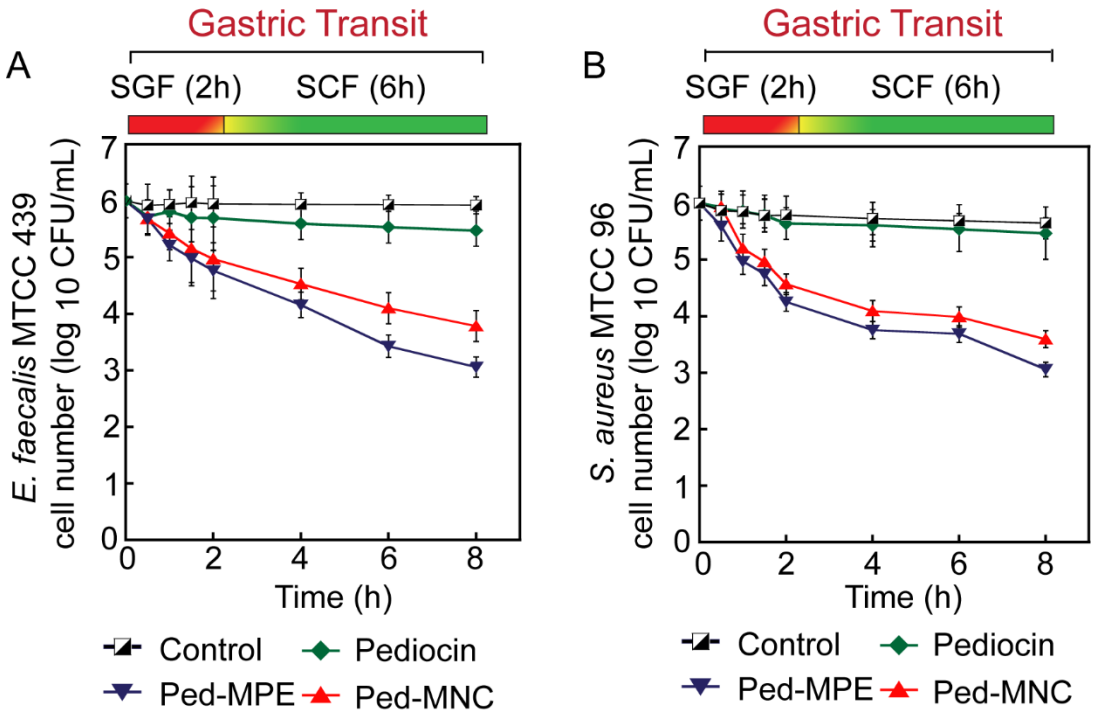

Figure S4. Effect of Ped-MPE and Ped-MNC on the viability of (A) *E. faecalis* MTCC 439 and (B) *S. aureus* MTCC 96 in a simulated gastric transit experiment.

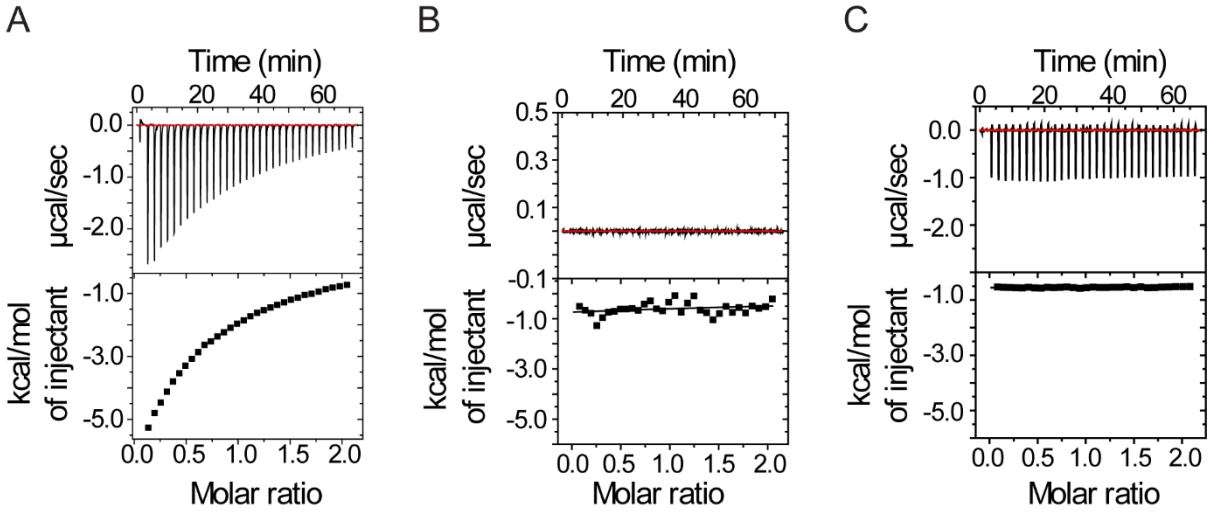

Figure S5. (A) Isothermal calorimetry (ITC) titration of (A) MPE (0.5 mg/mL) with SGF, (B) MNP (0.5 mg/mL) with solvent and (C) MPE (0.5 mg/mL) with solvent.

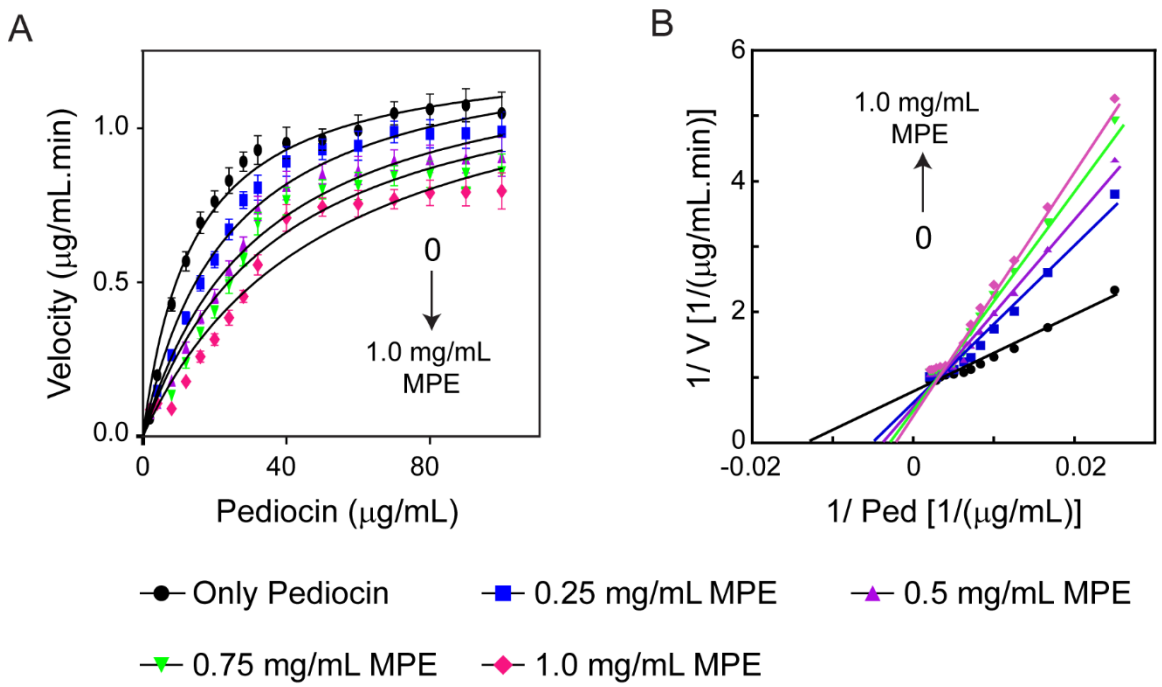

Figure S6. (A-B) Effect of MPE on the kinetics of pepsin-mediated digestion of pediocin.

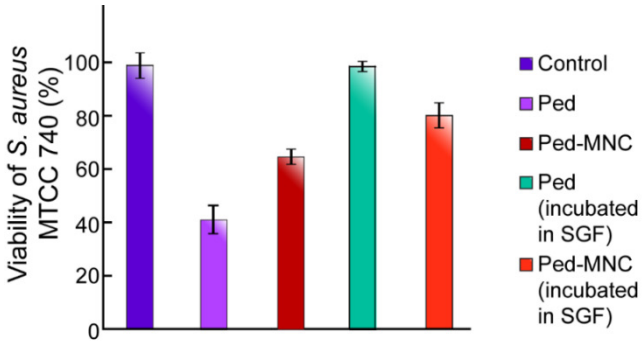

Figure S7. Effect of Ped-MNC on the viability of *S. aureus* MTCC 740 cells adhered onto HT-29 cell line.

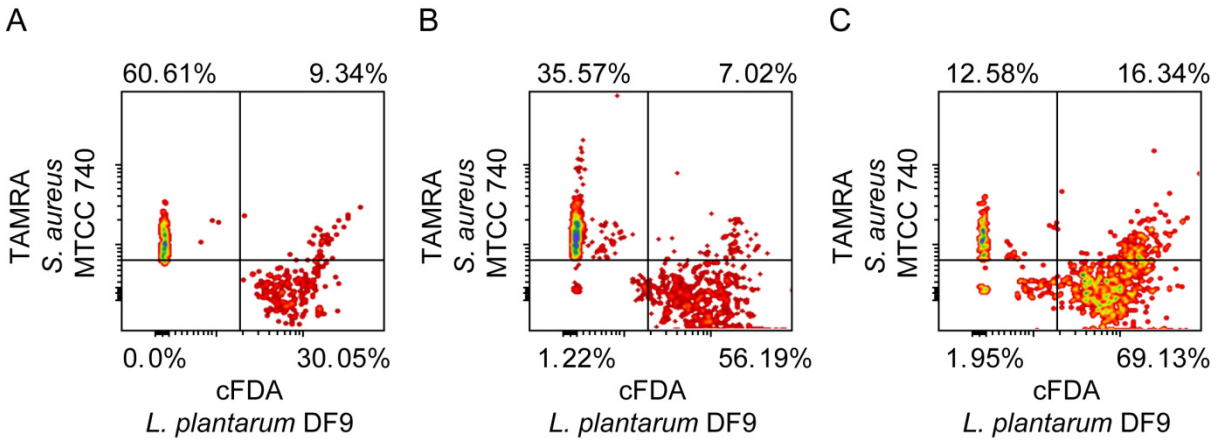

Figure S8. Dual color flow cytometry analysis to study the effect of (A) *L. plantarum* DF9, (B) *L. plantarum* DF9 and Ped-MNC and (C) *L. plantarum* DF9 and pediocin on the adhesion of *S. aureus* MTCC 740 onto HT-29 cells.

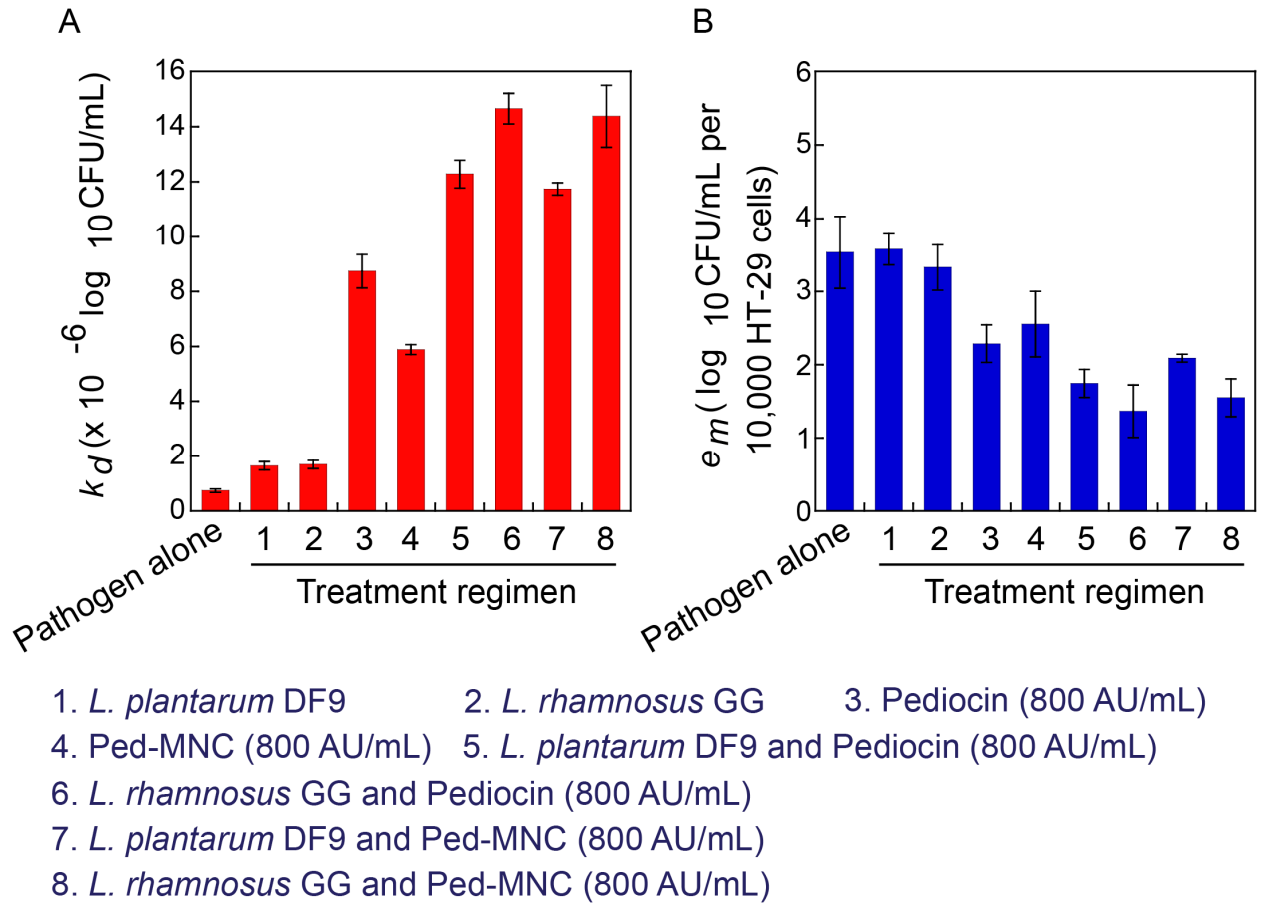

Figure S9. Effect of Ped-MNC in combination with probiotic LAB on the adhesion process parameters, (A) dissociation constant (k_d) and (B) maximum number (e_m) of *S. aureus* MTCC 740 cells adhered onto HT-29 cell line.