Bacteriocin-producing strains of *Lactobacillus plantarum* inhibit adhesion of *Staphylococcus aureus* to extracellular matrix: quantitative insight and implications in antibacterial therapy

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In the present study, the adhesion of bacteriocin-producing probiotic strains of *Lactobacillus plantarum* onto extracellular matrix (ECM) proteins such as collagen and mucin and their potential to prevent pathogen invasion onto the ECM was ascertained. Fluorescence-based *in vitro* assays indicated that *L. plantarum* strains CRA21, CRA38 and CRA52 displayed considerable adhesion to ECM molecules, which was comparable to the probiotic *Lactobacillus rhamnosus* GG. Flow cytometry-based quantitative assessment of the adhesion potential suggested that *L. plantarum* CRA21 exhibited superior adhesion onto the ECM as compared with other lactic acid bacteria strains. Furthermore, fluorescence-based assays suggested that the highest inhibition of *Staphylococcus aureus* adhesion onto collagen and mucin by bacteriocin-producing *L. plantarum* strains was observed in the exclusion mode as compared with the competition and displacement modes. This observation was supported by the higher binding affinity ($k_D$) for the ECM exhibited by the *L. plantarum* strains as compared with *S. aureus*. Interestingly, a crude plantaricin A extract from food isolates of *L. plantarum* displayed potent antibacterial activity on ECM-adhered *S. aureus* cells. It is envisaged that the *L. plantarum* isolates displaying bacteriocinogenic and ECM-adhering traits can perhaps be explored to develop safe antibacterial therapeutic agents.

**INTRODUCTION**

Lactic acid bacteria (LAB) constitute the cornerstone of food fermentation processes, and have been traditionally applied to develop a wide range of fermented food products owing to their significant contributions towards flavour, aroma and texture (Coolbear *et al.*, 2008; Leroy & De Vuyst, 2004). In recent times, LAB have also emerged as safe candidates for food safety applications and pathogen mitigation as several studies have demonstrated the inherent ability of LAB strains to produce antimicrobial peptides such as bacteriocins, which are known to act against bacterial pathogens (Cotter *et al.*, 2005, 2013; Drider *et al.*, 2006; Jagannath *et al.*, 2001; Jones *et al.*, 2008; O’Connor *et al.*, 2015; Singh & Ramesh, 2008; Singh *et al.*, 2012a). In addition, LAB have also come into the limelight as probiotic candidates, which can have a fundamental bearing on the activity and composition of the gut microbiota. In particular, *Lactobacillus rhamnosus* GG has been extensively studied, and is being marketed as a probiotic under the names ActifiPlus, Gefilus, LGG, Onaka He GG and VifitThus (Doron *et al.*, 2005; Saxelin *et al.*, 2005). Several literature reports have shown that LAB strains exhibit traits such as tolerance to low pH, digestive enzymes and bile (Begley *et al.*, 2006; De Vries *et al.*, 2006; Mukherjee *et al.*, 2013; Ouwehand *et al.*, 2002; Rastall *et al.*, 2005). These traits are likely to empower LAB strains during gastrointestinal transit. Furthermore, the ability of probiotic LAB to thrive in the intestinal niche and render significant health benefits is consolidated by their inherent propensity to adhere to intestinal cells, their antagonistic activity against pathogens known to invade the gastrointestinal tract, and their significant role in healing damaged mucosa and immunomodulation (Alander *et al.*, 1999; Collado *et al.*, 2007; Elliott *et al.*, 1998; Pagnini *et al.*, 2010; Reid & Burton, 2002; Zhou & Gill, 2005).

Apart from their conventional food applications, LAB are desirable as compelling scientific evidence seems to suggest that they can significantly impact the activity and composition of human intestinal microbiota (Preidis *et al.*, 2011;...
Shanahan, 2010). Based on this premise, probiotic LAB have been positioned in the vanguard of healthcare applications. Probiotic LAB can prevent pathogen colonization in the intestine by occupying sites and producing bacteriocins and other metabolites (Gopal et al., 2001; Lee et al., 2003; Millette et al., 2008; O’Shea et al., 2012). Several reports describe the beneficial effects of Lactobacillus species on the gastrointestinal tract (Bernbom et al., 2009; Guarner & Malagelada, 2003; O’Callaghan et al., 2012; Walter, 2008). Adhesion to the extracellular matrix (ECM) and intestinal cells is a key attribute of probiotic strains (Lebeer et al., 2010; Lee et al., 2004; Ouwehand & Salminen, 2003; Reid & Burton, 2002). Certain lactobacilli have been shown to have an inclination to adhere to ECM molecules such as collagen and mucin, and can thus be applied for inhibition of pathogen adhesion (Lorca et al., 2002; Miyoshi et al., 2006; Muñoz-Provenzio et al., 2009; Ouwehand et al., 2001; Vélez et al., 2007; Vesterlund et al., 2005; Yadav et al., 2013). Furthermore, mucin-binding lactobacilli have been shown to inhibit attachment of opportunistic pathogens and this is noteworthy as several enteric pathogens have evolved mechanisms to breach the mucosal barrier (McGuckin et al., 2011; Shanahan, 2010).

Amongst the gut-colonizing pathogens, emerging literature reports have elucidated the detrimental implications of colonization by Staphylococcus aureus (Acton et al., 2009; Yan et al., 2014). S. aureus is known to be equipped with adhesins known as microbial surface component-recognizing adhesive matrix molecules for binding to ECM molecules such as collagen (Foster et al., 2014). However, reports also suggest that binding to mucin is perhaps critical for S. aureus pathogenesis and persistence in the intestine (Gries et al., 2005; Vesterlund et al., 2006). Given this backdrop, there is a need to conceive alternate therapeutic approaches that could adequately combat colonization of the ECM by S. aureus. Although LAB have a long history of safe use in the food and agriculture industry, food isolates of LAB having probiotic traits also offer an exciting prospect of developing a safe LAB-based antibacterial regime, given their inherent propensity to survive in the harsh gastrointestinal niche and their ability to adhere to the ECM and host intestinal cells, and thereby prevent pathogen adhesion. Furthermore, it can be conceived that a bacteriocin-producing probiotic LAB may acquire a competitive advantage and constitute a dual armament as an anti-adhesion agent as well as a safe therapeutic antibacterial. Based on this premise, here we report the in vitro adhesion potential of bacteriocin-producing food isolates of Lactobacillus plantarum to ECM molecules collagen and mucin. The study entails quantitative and comparative assessment of the adhesion potential of the LAB strains onto collagen and mucin, and their subsequent application in preventing adhesion of the surrogate model pathogen S. aureus MTCC 96 to ECM molecules. The present study illustrates the prospect of exploring bacteriocin-producing LAB for potential mitigation of ECM colonization by pathogens.

**METHODS**

**Reagents and growth media.** 5 (and 6) carboxyfluorescein diacetate succinimidyel ester (CFDA-SE), SDS, Tween 20, collagen type I derived from human placenta, and mucin derived from porcine stomach were obtained from Sigma-Aldrich. Brain heart infusion (BHI) broth, de Man, Rogosa and Sharpe (MRS) broth, urea, sodium hydroxide, and crystal violet were procured from HIMedia.

**Bacterial strains.** The L. plantarum strains used in the present investigation were isolated previously from indigenous food sources, and characterized for their bacteriocinogenic and probiotic traits (Mukherjee et al., 2013; Singh et al., 2012a). The reference LAB, bacteriocin-producing LAB as well as non-LAB bacterial strains used in this investigation are listed in Table S1 (available in the online Supplementary Material). The strains were grown as per the conditions described in our previous investigation (Singh et al., 2012a).

**PCR-based detection of the collagen-binding protein (cnbp) gene in isolates of L. plantarum.** DNA was isolated from isolates of L. plantarum (isolates CRA21, CRA38, CRA49, CRA52 and DF9) by using the urea/SDS method as described previously (Singh & Ramesh, 2009). Detection of cnbp was accomplished using 5’-CGCTATTTA-TGCTGAGGATGAC-3′ as forward primer and 5’-GCCCTACGT-ATCTGTAACCAC-3′ as reverse primer. The PCR mix consisted of PCR buffer (diluted from a 10× stock), 200 μM each dNTP, 1 U Taq DNA polymerase (Bioline), and 50 pmol each forward and reverse primer. The template DNA was initially subjected to denaturation at 94°C for 2 min followed by a total of 35 amplification cycles in a programmable thermal cycler (Gene Amp Gold PCR System; Applied Biosystems). Each cycle included denaturation for 1.0 min at 94°C, primer annealing for 1.0 min at 56°C and extension for 1.0 min at 72°C. A final extension at 72°C for 10 min followed the last cycle. The PCR products were subjected to agarose (1% w/v) gel electrophoresis.

**Comparative assessment of adhesion of L. plantarum strains to collagen and mucin.** Collagen and mucin binding was performed as described previously (Muñoz-Provenzio et al., 2009). A stock solution of collagen was prepared in filter-sterilized PBS (pH 5.5) to a final concentration of 1.0 mg ml⁻¹ and stored at −20°C prior to experiments. A stock solution of mucin was prepared in 0.1 M acetate buffer (pH 5.0) to a final concentration of 1.0 mg ml⁻¹ and stored at 4°C prior to experiments. Briefly, 96-well microtitre plates (Maxisorb; Nunc) were coated with collagen and mucin overnight at 4°C at concentrations of 50 and 500 μg ml⁻¹, respectively. Following coating, the wells were washed with sterile PBS three times and blocked with 0.1% (v/v) Tween 20 for 1 h. The adhesion potential of L. plantarum strains to collagen and mucin was studied by crystal violet staining, fluorescence-based adhesion assay and flow cytometry, as described in the following sections.

**Crystal violet staining.** The reference and LAB strains were grown overnight, harvested by centrifugation, washed and resuspended in sterile PBS to OD₆₀₀ 1.0. Subsequently, 100 μl cells was added to the collagen- or mucin-coated wells and incubated overnight at 4°C. Non-adhered cells were aspirated from the wells and the wells were washed three times with sterile PBS and 0.05% (v/v) Tween 20, and air-dried in a laminar hood. Crystal violet was then added to the wells at a final concentration of 1.0 mg ml⁻¹ and incubated for 45 min. The dye solution was aspirated, followed by three PBS washes, and subsequently 50 mM citrate buffer (pH 4.0) was added to the wells and incubated for 45 min. Abs₅₉₅ of the solution was measured in a multilplate reader (Infinite M200; Tecan). To quantify the adhesion ability of the selected LAB strains as compared with L. rhamnosus GG, the absorbance obtained for the tested strain was compared with that obtained in the case of L. rhamnosus GG and expressed as percentage

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adhesion potential as compared with *L. rhamnosus* GG. All the experiments were performed in triplicates and mean ± SD was calculated. Statistical analysis was performed by one-way ANOVA using Sigma Plot version 11.0.

**Fluorescence-based adhesion assay.** The reference and LAB strains were grown overnight, harvested by centrifugation, washed and resuspended in sterile PBS to OD_{600} 1.0. Fluorescence labelling of LAB strains with cFDA-SE was accomplished as described previously (Singh et al., 2012a). The solution fluorescence of a 100 µl aliquot of the cFDA-SE-labelled LAB strains was measured in a multiplate reader (Infinite M200) and considered as total fluorescence (F_t). Subsequently, 100 µl aliquots of cFDA-SE-labelled LAB cells were added to collagen- or mucin-coated wells and incubated overnight at 4 °C. Following incubation, non-adhered cells were aspirated and their fluorescence intensity was measured (F_{NA}). A quantitative measure of LAB adhesion (F_t - F_{NA}) to collagen and mucin was obtained by ascertaining the difference between total fluorescence (F_t) and fluorescence intensity for non-adhered cells (F_{NA}). To quantify the adhesion ability of the selected LAB strains as compared with *L. rhamnosus* GG, F_{t} of the tested strain was compared with that obtained in the case of *L. rhamnosus* GG and expressed as percentage adhesion potential as compared with *L. rhamnosus* GG. The adhered cells were also observed under a fluorescence microscope (Eclipse Ti-U; Nikon) with a filter that allowed blue light excitation for cFDA-SE. All the experiments were performed in triplicates and mean ± SD was calculated. Statistical analysis was performed by one-way ANOVA using Sigma Plot version 11.0.

**Flow cytometry analysis of adhesion to ECM.** The adhesion of *L. rhamnosus* GG and the strains of *L. plantarum* to collagen and mucin was evaluated with the help of flow cytometry, which was performed on a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems) equipped with a 15 mW, 488 nm, air-cooled argon ion laser. LAB cells were grown for 6 h, harvested and labelled with cFDA-SE as described previously (Singh et al., 2012a). A 100 µl aliquot of cFDA-SE-labelled bacterial cells (10^6 c.f.u. ml^{-1}) was then incubated overnight at 4 °C in microtitre plate wells coated with collagen or mucin. In another set of microtitre plate wells devoid of collagen or mucin, cFDA-SE-labelled bacteria were incubated for the same period of time and considered as control cells. Subsequently, the supernatant containing non-adhered cells was aspirated from both sets of wells and diluted in 1.0 ml filter-sterilized PBS (pH 7.4). The aspirated cells were subjected to flow cytometry analysis at a low flow rate setting and the instrument was adjusted to acquire 30 000 events. Unlabelled cells were used to compensate for cellular autofluorescence and to set the appropriate voltage and threshold parameters. Forward-angle light scatter versus side scatter plots were analysed to detect bacterial cells. Detection of green fluorescence of cFDA-stained cells was accomplished through the FL1 channel (band pass filter 530 nm). Data acquisition was performed with CellQuest Pro 6.0 (Becton Dickinson). Data analysis was performed with the assistance of FCS Express 5.0 (DeNovo).

**Dose-dependent adhesion of *L. plantarum* strains to collagen and mucin.** *L. rhamnosus* GG, *L. plantarum* strains CRA21, CRA38 and CRA52, and *S. aureus* MTCC 96 were grown overnight, harvested and labelled with cFDA-SE as described earlier. To ascertain the dose-dependent adhesion of the strains, 100 µl aliquots of cFDA-SE labelled cells of the bacterial strains were added in the range of 2.0–8.0 log_{10} c.f.u. per well to collagen- or mucin-coated 96-well microtitre plate wells and a fluorescence-based adhesion assay for each cell concentration was performed as described earlier. The solution fluorescence of a 100 µl aliquot of the cFDA-SE-labelled LAB strains of varying cell number (2.0–8.0 log_{10} c.f.u. per well) was measured in a multiplate reader (Infinite M200) and considered as total fluorescence (F_t). For each LAB strain, the dose-dependent adhesion onto collagen- or mucin-coated wells was quantified by determining F_t [difference between total fluorescence (F_t) and fluorescence intensity for non-adhered cells (F_{NA}) for every cell number used in the adhesion assay]. Subsequently, for every LAB strain and at each cell number, adhesion onto collagen- or mucin-coated wells was ascertained by comparing F_t and F_{NA} values and expressing the results as per cent adhesion [(F_t/F_{NA}) × 100]. The maximum number of adhered bacteria (F_{max}) and the dissociation constant (k_d) for the adhesion process were ascertained by the method described previously (Lee et al., 2000). All the experiments were performed in triplicates and mean ± SD was calculated.

**Inhibition of *S. aureus* MTCC 96 adhesion to ECM by *L. plantarum*.** Cells of *L. plantarum* strains CRA21, CRA38 and CRA52 and *L. rhamnosus* GG were grown in MRS, and cells of *S. aureus* MTCC 96 were grown in BHI broth for 6 h each. The cells were then harvested by centrifugation, washed and resuspended in sterile PBS to OD_{600} 1.0. Cells of *S. aureus* MTCC 96 were then labelled with cFDA-SE following the protocol described previously (Singh et al., 2012a) and fluorescence intensity was measured (F_t) using a multiplate reader (Infinite M200). To determine the ability of LAB strains to inhibit *S. aureus* adhesion, three experimental models, i.e. competition, displacement and exclusion, were adopted. In the displacement mode, cFDA-SE-labelled *S. aureus* MTCC 96 cells in PBS (6.0 log_{10} c.f.u. per well) were added to 96-well microtitre plates (Maxisorp) coated with either collagen (50 µg ml^{-1}) or mucin (500 µg ml^{-1}) and incubated for 1 h. The wells were then washed with sterile PBS to remove the non-adherent bacteria. Subsequently, LAB cells (6.0 log_{10} c.f.u. per well) were added to the wells and incubated for 1 h. In the case of exclusion, LAB cells (6.0 log_{10} c.f.u. per well) were first added to ECM-coated wells and incubated for 1 h, followed by washing and removal of non-adherent LAB by sterile PBS. Subsequently, cFDA-SE-labelled cells of *S. aureus* MTCC 96 (6.0 log_{10} c.f.u. per well) were added to collagen- or mucin-coated wells bearing adherent LAB and further incubated for 1 h. In competitive inhibition assays, LAB cells as well as cFDA-SE-labelled cells of *S. aureus* MTCC 96 (6.0 log_{10} c.f.u. per well for each) were added simultaneously to ECM-coated wells and incubated for 2 h. The non-adherent *S. aureus* cells from all the experimental samples were aspirated from ECM-coated wells and their fluorescence intensity (F_{NA}) was measured using a multiplate reader (Infinite M200). Adhesion of *S. aureus* for each experimental setup in collagen- and mucin-coated wells was determined by calculating the difference between F_t and F_{NA}, respectively. The results were represented as percentage inhibition by comparing the adhesion obtained for each mode of inhibition with that obtained without addition of any *L. plantarum* cells. All the experiments were performed in triplicates and mean ± SD was calculated. The adhered cFDA-SE-labelled *S. aureus* cells in the three adhesion modes were also observed under a fluorescence microscope (Eclipse Ti-U) with a filter that allowed blue light excitation for cFDA-SE.

**Effect of plantaricin A extract on *S. aureus* MTCC 96 cells adhering on collagen and mucin.** Initially, bacteriocin-producing *L. plantarum* strains CRA21, CRA38 and CRA52 were grown overnight in 10.0 ml sterile MRS broth, followed by centrifugation to separate the bacterial cells. The supernatant was then extensively dialysed in a 1.0 kDa molecular mass cut-off dialysis bag, followed by freeze drying and reconstitution in 1.0 ml sterile PBS. The reconstituted fraction was considered as a crude plantaricin A extract. The bacteriocin activity in this extract was ascertained by the spot-on-lawn method and expressed as arbitrary units (AU) ml^{-1} as described previously (Singh et al., 2012a). Prior to the assay, cells of *S. aureus* MTCC 96 were labelled with cFDA-SE following the protocol described previously (Singh et al., 2012a) and fluorescence intensity was measured (F_t) using a multiplate reader (Infinite M200). Subsequently, the cells were allowed to adhere onto collagen- or mucin-
coated wells for 2 h followed by aspiration of non-adhered cells and washing to remove sparsely adhered cells. To each well, a crude plantaricin A extract obtained from the L. plantarum strains was added at a final concentration of 400 AU ml\(^{-1}\). Following incubation for 2 h, the supernatant was gently aspirated, centrifuged to remove any cells and the fluorescence intensity of the collected supernatant \((F_s)\) was measured using a multispot reader (Infinite M200). The results were expressed as percentage leakage by comparing \(F_s\) to \(F_b\) in the experiments involving plantaricin A extract from each L. plantarum. All the experiments were performed in triplicates and mean \(\pm SD\) was calculated. The adhered cFDA-SE labelled S. aureus cells following treatment with plantaricin A extract were also observed under a fluorescence microscope (Eclipse Ti-U) with a filter that allowed blue light excitation for cFDA-SE.

**Nucleic acid sequence.** The PCR-generated amplicon for cnbp gene of L. plantarum CRA38 was sequenced and subsequently subjected to BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The partial coding sequence was deposited in GenBank with accession number JQ231230.1.

### RESULTS AND DISCUSSION

#### Detection of cnbp in bacteriocin-producing L. plantarum strains

In a previous study, L. plantarum strains were isolated from various indigenous sources and were characterized as potent bacteriocin producers with probiotic potential (Mukherjee et al., 2013; Singh et al., 2012a). With an aim to expand the application potential of these strains, it was envisaged that determination of the adhesion potential of the strains to the ECM would be pertinent, as adhesion to ECM molecules such as collagen and mucin is considered as a cardinal feature of probiotic strains (Lebeer et al., 2008). The ability of the pathogen S. aureus to colonize the intestine in conjunction with its ability to adhere to ECM molecules is a serious healthcare concern and has been reported to be responsible for various ailments (Acton et al., 2009; Bhalla et al., 2007; Foster et al., 2014; Gries et al., 2005; Hansen et al., 2006). In this context, probiotic LAB strains having a propensity to adhere onto the ECM such as collagen and mucin could bear interesting therapeutic potential to prevent invasion of the ECM by S. aureus. In this endeavour, the initial objective was to determine the presence of genes coding for specific ECM-binding proteins such as collagen-binding protein (cnbp) in L. plantarum strains by PCR. All the L. plantarum strains yielded amplicons of expected size in PCR using cnbp gene-specific primers (Fig. 1). The size of the amplicons obtained from the L. plantarum strains coincided with that of the standard L. plantarum strains (Fig. 1). The cnbp amplicon of L. plantarum CRA38 was sequenced and assigned a GenBank accession number JQ231230.1. BLAST analysis of the nucleotide sequence indicated \(\sim 99\ %\) homology with previously deposited cnbp gene sequences of L. plantarum strains. The presence of cnbp suggested the potential of L. plantarum strains to adhere onto collagen. Collagen-binding protein of L. plantarum has been previously reported to be instrumental in adhesion and in reducing the binding of gut pathogens to collagen (Yadav et al., 2013).

#### Adhesion to collagen and mucin by L. plantarum strains

In order to validate the potential of the L. plantarum strains to adhere onto the ECM, conventional in vitro binding assays with collagen and mucin were performed. Prior to the binding assays, the L. plantarum strains were labelled with the fluorescent dye cFDA-SE, based on previous investigations, which established that cFDA labelling of LAB strains retained the viability of the cells and was also suitable for performing in vitro adhesion assays (Fuller et al., 2000; Mukherjee et al., 2013). For the adhesion assays, L. rhamnosus GG was selected as a standard probiotic strain (Doron et al., 2005) for comparative evaluation. As observed in Fig. 2(a), collagen-binding assays revealed that the strains L. plantarum CRA21, CRA38 and CRA52 displayed high in vitro adhesion to human collagen type I (95–97\ % adhesion potential) and this was on a par with the standard strain L. rhamnosus GG whose adhesion potential was considered to be 100\ %. On comparison, it was also observed that L. plantarum DF9 exhibited considerably lower adhesion potential to collagen as evident from Fig. 2(a). The trend of adhesion to collagen exhibited by the L. plantarum strains was corroborated by crystal violet staining as shown in Fig. 2(b). Interestingly, the LAB strains L. plantarum CRA21, CRA38 and CRA52, which had earlier displayed appreciable adhesion to collagen, were also observed to exhibit high in vitro adhesion potential to mucin, comparable with that observed for L. rhamnosus GG and L. plantarum MTCC 1407 as depicted in Fig. 2(c). Amongst the L. plantarum strains, CRA49 and DF9 revealed inferior adhesion potential to mucin. The overall adhesion potential of L. plantarum strains to mucin ascertained by the fluorescence-based method was also substantiated by crystal violet staining as shown in Fig. 2(d). In the context of collagen and mucin adhesion potential, the minor variations in the results obtained in the fluorescence-based assay and crystal
The violet staining method could perhaps be attributed to the varying sensitivity of the methods as well as the inherent differences in ECM binding amongst the strains, which is captured in the results. In the present study, cFDA-SE labelling provided a convenient analytical tool and enabled tracking of the adhesion of *L. plantarum* strains onto collagen and mucin. The manifestation of bright green fluorescent cells of the *L. plantarum* strains adhering onto the selected ECM as shown in Fig. 3(a–h) provided additional qualitative evidence for their adhesion.

The adhesion of LAB strains onto collagen and mucin was also investigated by flow cytometry. In these experiments, the LAB strains were labelled with cFDA-SE prior to adhesion assays. From the contour plots obtained in flow cytometry, the essential observation was that the median of the cell population shifted when the non-adhered cells were analysed as compared with the initial cells (total cells) that were subjected to adhesion on collagen and mucin (Fig. 4). This observation was unequivocal for the LAB strains tested and was quantitatively verified by

![Figure 2](image-url)

**Fig. 2.** Adhesion of *Lactobacillus* spp. and *L. plantarum* strains onto (a, b) collagen and (c, d) mucin: results from fluorescence-based assay (a, c) and results based on crystal violet assay (b, d). *Significant difference based on ANOVA (P<0.05).
statistical analysis through comparison of the median for the cell populations (Table S2). Collectively, the shift in the median in contour plots (Fig. 4) in conjunction with quantitative analysis of the median (Table S2) reflected the propensity of the bacteriocinogenic \(L.\) \(\text{plantarum}\) strains to adhere onto collagen and mucin, and corroborated the earlier evidence on adhesion of the tested strains on the selected ECM molecules.

**Quantitative analysis of binding to collagen and mucin**

Following assessment of the adhesion potential of \(L.\) \(\text{plantarum}\) strains on collagen and mucin, the next objective was to perform a comparative and quantitative appraisal of the in vitro adhesion of \(L.\) \(\text{plantarum}\) strains onto the ECM and to determine their binding affinities. On the basis of their adhesion potential on the ECM, \(L.\) \(\text{plantarum}\) strains CRA21, CRA38 and CRA52 were selected for these experiments along with the reference probiotic \(L.\) \(\text{rhamnosus}\) GG. A fluorescence-based adhesion assay on collagen and mucin indicated a systematic increase in adhesion for all the tested strains, with increasing cell concentrations up to \(6.0 \log_{10}\text{c.f.u. per well}\) (Fig. 5). At higher cell concentrations (\(7.0 \text{ and } 8.0 \log_{10}\text{c.f.u. per well}\)), the increase in adhesion was nominal, which suggested a saturation effect (Fig. 5). As evident from Fig. 5(a), at the highest initial cell concentration of \(8.0 \log_{10}\text{c.f.u. per well}\), the maximum adhesion on collagen was demonstrated by \(L.\) \(\text{rhamnosus}\) GG (48 %) followed by the \(L.\) \(\text{plantarum}\) strains CRA21 (31 %), CRA38 (25 %) and CRA52 (24 %), respectively. An analogous trend was also observed in the case of adhesion on mucin as shown in Fig. 5(b). When the reciprocal plots (reciprocal of adhered bacteria cell number plotted against the reciprocal of the number of added bacterial cells) were analysed, a linear relationship emerged in the case of \(L.\) \(\text{rhamnosus}\) GG (Fig. S1). However, in the case of the \(L.\) \(\text{plantarum}\) strains, two distinct linear sections could be identified (Fig. S1). This observation suggested that the \(L.\) \(\text{plantarum}\) strains perhaps display two exclusive modes of binding to collagen and mucin. Presumably, one of them represents adhesion for a high bacterial cell number, which may be attributed to non-specific descriptors such as van der Waals and hydrophobic interactions. Conversely, the other linear region of the plot accounts for adhesion to collagen and mucin at lower cell concentrations, which is largely represented by a specific adhesion mechanism. This bimodal phenomenon of adhesion onto the ECM by LAB strains has been described in previous studies (Lee et al., 2000; Vesterlund et al., 2006).

The comparative adhesion potential of the LAB strains onto the ECM was also captured through estimation of quantitative parameters such as maximum number of bacteria bound to ECM \((e_m)\) and dissociation constant \((k_d)\). In the case of adhesion to collagen, the salient observation was that the maximum number of adhered cells for all the LAB strains was comparable (Table 1). Furthermore, the dissociation constant of the tested LAB strains for the adhesion process onto collagen was also observed to be by and large equivalent, and was on a par with the standard \(L.\) \(\text{rhamnosus}\) GG strain (Table 1). In the case of mucin, the maximum number of adhered cells was observed to be highest for \(L.\) \(\text{rhamnosus}\) GG (1.78 \(\log_{10}\text{c.f.u. per well}\), etc.)
**Fig. 4.** Flow cytometry analysis of adhesion of LAB strains onto collagen and mucin: (a–c) *L. rhamnosus* GG, (d–f) *L. plantarum* CRA21, (g–i) *L. plantarum* CRA38 and (j–l) *L. plantarum* CRA52.
whilst it decreased progressively for the *L. plantarum* strains. Interestingly, the dissociation constant for the adhesion process to mucin was observed to be lowest for *L. plantarum* CRA52 ($k_d=7.58 \log_{10}$ c.f.u. per well), which suggested that this strain exhibited superior adhesion to mucin as compared with the other tested LAB strains.

**In vitro inhibition of *S. aureus* adhesion to ECM by *L. plantarum* strains**

The results obtained in the comparative adhesion assays were encouraging and suggested that the *L. plantarum* strains could perhaps be explored in inhibiting adhesion of model gut pathogens to the ECM. To this end, standard *in vitro* adhesion assays on collagen and mucin were conducted in the three formats, i.e. exclusion, competition and displacement. As observed in Fig. 6(a), a fluorescence-based method revealed that *L. rhamnosus* GG, and *L. plantarum* CRA21, CRA38 and CRA52 strains could exclude *S. aureus* MTCC 96 from binding to collagen. Interestingly, amongst the LAB strains, *L. plantarum* CRA21 showed considerable inhibition of adhesion of *S. aureus* cells (85%), which was on a par with that of the standard probiotic *L. rhamnosus* GG. In the exclusion mode, addition of LAB onto collagen-coated wells preceded addition of *S. aureus* cells. Based on the strong adhesion parameters of *L. plantarum* strains onto collagen (Table 1), it is anticipated that these strains would exhibit efficient colonization of the ECM, which perhaps resulted in the reduced ability of *S. aureus* cells to adhere onto collagen. It can be presumed that the ability of the *L. plantarum* strains to prevent adhesion of *S. aureus* cells onto collagen is also likely to be governed by a combinatorial effect of the adhesion process parameters ($e_m$ and $k_d$). In the exclusion model, it can also be conceived that the initial strong adhesion of LAB cells onto collagen as reflected by the low dissociation constant ($k_d$) of the adhesion process could be critical for efficient exclusion of *S. aureus* added subsequently. The differences observed in the ability to exclude *S. aureus* adhesion onto collagen by the bacteriocin-producing *L. plantarum* strains may be attributed to the differences in their inherent binding parameters ($e_m$ and $k_d$), which could be captured in the experiments (Table 1). Furthermore, in the exclusion mode, the probability of *S. aureus* cells adhering onto collagen would be largely governed by the ability to displace initially colonizing LAB. This phenomenon, in turn, would depend on the magnitude of the binding affinity of *S. aureus* with collagen ($k_d$). To this end, it was observed that in the case of collagen, the dissociation constant for *S. aureus* MTCC 96 binding with collagen ($k_d=12.85 \log_{10}$ c.f.u. per well) was distinctly higher (Table S3) than that obtained in the case

**Table 1.** Quantitative parameters for the adhesion process of LAB strains on collagen and mucin

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$e_m$, Maximum number of adhered bacteria; $k_d$, dissociation constant.
of the *L. plantarum* strains. This perhaps provides a plausible explanation for the strong inhibition of *S. aureus* adhesion on collagen initially colonized by LAB strains. A similar phenomenon was also observed in the case of inhibition of *S. aureus* adhesion onto mucin by LAB strains in the exclusion mode as shown in Fig. 6(b). However, the inhibition of *S. aureus* adhesion onto mucin was comparatively less than that observed in the case of collagen in the exclusion mode. The scope of competitive inhibition of *S. aureus* adhesion in collagen- or mucin-coated wells by LAB strains was also studied. As shown in Fig. 6(a), the magnitude of inhibition of *S. aureus* adhesion in these interactions was observed to be highest for *L. rhamnosus* GG (69 %) followed by *L. plantarum* CRA21 (62 %), CRA38 (60 %) and CRA52 (58 %). In the case of competition, the dissociation constant ($k_d$) presumably is pivotal as it determines the binding affinity of the cells with the ECM molecules collagen and mucin. Although, the higher $e_m$ values for *S. aureus* (Table S3) as compared with LAB strains (Table 1) imply higher probability of ECM coverage,

![Fig. 6](image-url). Inhibition of *S. aureus* MTCC 96 adhesion onto (a) collagen and (b) mucin by *L. plantarum* strains. (c) Fluorescence microscopy analysis to study the inhibition of *S. aureus* adhesion onto (i–iv) collagen and (v–viii) mucin by *L. plantarum* CRA21. Bar, 200 μm.
the prominent difference in the dissociation constant ($k_d$) obtained for the LAB strains as compared with S. aureus cells (Tables 1 and S3) perhaps has an overriding effect and accounts for the ability of the L. plantarum strains to outcompete S. aureus significantly in the adhesion assays conducted in competition mode. A similar phenomenon was observed in the competition mode in the case of mucin. In the displacement mode of adhesion on collagen or mucin, it was observed that the inhibition of adhesion of S. aureus cells to ECM-coated wells was considerably lower than that of the other tested interactions (Fig. 6a, b). Initial colonization of ECM by S. aureus is effective as the bacterium is able to successfully occupy the bulk of the binding sites. Subsequent addition of LAB strains is less effective to abrogate the high surface coverage of the ECM by S. aureus cells owing to the reduced ability of the added LAB cells to displace S. aureus cells – a phenomenon that can be attributed to the inferior $w_o$ obtained for the LAB strains as compared with S. aureus (Tables 1 and S3). Fluorescence microscopy analysis revealed that a large number of cFDA-SE-labelled S. aureus MTCC 96 cells could adhere onto the ECM (Fig. 6c, i and v). Interestingly, in the presence of L. plantarum CRA21, adhesion of S. aureus MTCC 96 cells onto the ECM was observed to be reduced in the three tested models of adhesion assay (Fig. 6c, ii–iv and vi–viii). Note that an analogous trend of inhibition of adhesion by the L. plantarum strains in the three tested formats was also observed in the case of the model target bacteria Listeria monocytogenes Scott A and Enterococcus faecalis MTCC 439 (Fig. S2).

Effect of bacteriocin extract from L. plantarum strains on ECM-adhering target bacteria

Cumulatively, the results obtained in the adhesion assays illustrated the potential of the LAB isolates in inhibiting adhesion of S. aureus to the ECM molecules collagen and mucin. The food isolates of L. plantarum used in the present study were earlier characterized as potent plantaricin A producers displaying activity against the target bacteria S. aureus MTCC 96, L. monocytogenes Scott A and E. faecalis MTCC 439 (Singh et al., 2012a). Literature reports suggest that these target bacteria are known to colonize the ECM and are implicated in disruption of ECM-related barrier functions (Gries et al., 2005; Singh et al., 2012b; Flemming & Ackermann, 2007). As the bacteriocin plantaricin A produced by the L. plantarum strains exhibited profound activity against the target bacteria S. aureus MTCC 96, L. monocytogenes Scott A and E. faecalis MTCC 439 (Singh et al., 2012a), it was envisaged that treatment with plantaricin A would likely result in a significant loss of viability of these bacterial strains, which in turn would curtail their ability to adhere to and colonize the ECM. Thus, plantaricin A secreted by the L. plantarum strains could perhaps be explored as a safe antibacterial agent to mitigate adhesion or eradicate pre-colonized cells of S. aureus, L. monocytogenes and E. faecalis from the ECM. To this end, the surrogate model strains were allowed to adhere onto collagen- or mucin-coated wells in separate sets, followed by addition of a crude sample of plantaricin A extract (400 AU ml$^{-1}$) from the LAB strains CRA21, CRA38 and CRA52, and incubated for 2 h. In these experiments, the target bacterial cells were pre-labelled with the fluorescent dye cFDA-SE, which enabled us to pursue a fluorescence-based dye leakage assay to ascertain the effect of the bacteriocin on ECM-adhered cells. Interestingly, as observed in Fig. 7(a), exposure of collagen-adhering bacterial cells to plantaricin A extracts from the food isolates of L. plantarum resulted in notable leakage of cFDA-SE, which perhaps originated from the signature membrane-directed activity of plantaricin A (Singh et al., 2012a). Upon treatment with the plantaricin A extract obtained from the LAB strains, the maximum extent of dye leakage was observed for L. monocytogenes, which was anticipated given the strong antilisterial activity associated with the bacteriocin (Singh et al., 2012a). From Fig. 7(a), it can also be observed that amongst the LAB strains, plantaricin A extract obtained from L. plantarum CRA52 displayed the highest antilisterial activity on collagen-adhered cells as evident from the high dye leakage (38.52 %) as compared with the results obtained with plantaricin A extracts from L. plantarum CRA21 and CRA38, wherein the dye leakage amounted to 32.77 and 19.26 %, respectively. As evident in Fig. 7(b), fluorescence microscopy analysis of cFDA-SE-labelled target bacterial cells adhering onto collagen and treated with plantaricin A extracts from L. plantarum strains indicated a reduction in the cell number in the case of bacteriocin-treated sample and these results were consistent with the trend observed in the dye leakage assays depicted in Fig. 7(a). It is also significant that the overall trend of plantaricin A-mediated abrogation of target bacterial cells adhering onto collagen could be captured in experiments conducted with mucin (Fig. S3). The results obtained in these experiments are particularly encouraging considering a previous report that describes the critical role of plantaricin A as a pheromone that promotes biofilm formation and adhesion of L. plantarum strains onto the Caco-2 cell line, and thereby prevents pathogen adhesion onto the cells (Calasso et al., 2013). In light of this study, it would perhaps be interesting in future to determine the biofilm-forming potential of plantaricin A-producing L. plantarum strains and their effect in precluding adhesion of target pathogens onto the ECM.

In the present investigation, it was observed that bacteriocin-producing food isolates of L. plantarum essentially demonstrated a natural proclivity to adhere to the ECM molecules collagen and mucin. Empowered with bacteriocinogenic and putative probiotic traits, the prominent ECM-adhering L. plantarum isolates emerge as promising strains having a competitive edge, whose potential can perhaps extend from the conventional domain of food fermentation to potent healthcare applications. Considering the food origin of these strains, it is conceivable that the L. plantarum strains could be readily explored to develop food formulations tailored towards healthcare benefits. In future, it would be important to validate this scope of
the food isolates in in vivo models as a step towards the rational development of LAB-based safe oral food formulations that are increasingly being considered as radical agents for niche-specific antibacterial therapy.

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