CHAPTER 7

Binding of Acridine orange by Probiotic *Lactobacillus rhamnosus* Strains of Human Origin

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Introduction

Lactobacilli and their fermented products are thought to confer a variety of nutritional and therapeutic benefits, including antimutagenic and anticarcinogenic properties (Fernandes *et al*., 1987). Lactic acid bacteria and their metabolites inhibit mutagenicity and carcinogenicity of a range of chemical compounds found in the human diet, such as amino acid pyrolysates formed in cooked meat (Rafter, 1995). Although the mechanisms of antimutagenicity and anticarcinogenicity are unclear, such bacteria are proposed to decrease absorption of mutagens and carcinogens in the small intestine by bacterial binding (Terahara *et al*., 1998). Previous studies reported high correlation between antimutagenicity and mutagen binding properties, suggesting inactivation of chemicals upon binding (Sreekumar and Hosono, 1998). The cell wall components in *Streptococcus faecalis* that engender antimutagenic activity are assumed to be carbohydrate, carbohydrate complex, or lipid-carbohydrate complex (Hosono *et al*., 1988). The protective effect of lactic acid bacteria against food mutagens such as heterocyclic amines, *N*-nitroso compounds, and aflatoxins has been reported (Hosoda *et al*., 1996; Orrhage *et al*., 1994; Sreekumar and Hosono, 1998; Zhang and Ohta, 1990). These studies have proposed physical binding as one of the mechanisms of mutagen removal by *Lactobacillus* strains.

Acridines and their derivatives are used in enormous quantities in both medicine and industry. Soluble acridine dyes are utilized as pigments and dyestuff to color materials such as crude vegetable fibers, leather and cellulose. Acridines also find uses in industrial disinfection and preservation of metals and as antioxidants and corrosion inhibitors. Acridine compounds and their derivatives form complexes with DNA by intercalation and can exert the variety of biological effects. Acridines are also shown to cause frameshift mutations of both the types, addition and deletion (Nasim & Brychcy, 1979).

Lactobacillus rhamnosus Fb and Vc strains used in the present study possess probiotic properties: (i) acid-bile-phenol, and NaCl tolerance, (ii) are non-pathogenic, non-

haemolytic, and DNAase negative, (iii) exhibit mucin adherence, autoaggregation and coaggregation abilities, (iv) exhibit broad antimicrobial spectrum against human pathogens and food-spoilage organisms, (v) produce β-galactosidase activity, (vi) are susceptible to a range of antibiotics making the strains safer for their use as probiotics, and (vii) lyophilized powder of the cells retain probiotic properties and can act as a functional ingredient in the formulation of probiotic products (Pithva *et al*., 2014). Moreover, the strains exhibit antigenotoxic and antimutagenic activities against *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (Unpublished results). This study focuses on the binding of acridine orange (AO) by these probiotic *Lactobacillus rhamnosus* strains.

Materials and Methods

Bacterial strains and mutagen

Lactobacillus strains used in this study were obtained from different sources, which include infant fecal isolate *Lactobacillus rhamnosus* Fb JX406746 and vaginal isolate of healthy female *Lactobacillus rhamnosus* Vc JX406745. Reference strains *L. rhamnosus* GG (ATCC 53103), *L. rhamnosus* 231 (EF661653) (Ambalam *et al*., 2011; 2014) and *L. casei* DN 114 001 (Actimel strain, DANONE, France) were also included in the study. *Lactobacillus* strains were grown in de Man-Rogosa-Sharpe (MRS; Himedia, Mumbai, India) medium for 24 h at 37°C. The stock cultures were maintained in 10% skim milk at 4°C. Acridine orange (CAS No. 10127-02-3) was purchased from Himedia (Mumbai, India). Stock solution of Acridine orange (50 mg/ml) was prepared in dimethyl sulfoxide (DMSO) and stored at -20°C. Working solutions were prepared from the stock solution as and when required. Dilutions ranging from 10 - 100 µg/ml were used to prepare standard curve to determine the concentration of residual acridine orange in the reaction mixtures.

Binding assay with Acridine orange by non-growing cells

The cells were obtained from 24 h cultures of lactobacilli grown in MRS medium by centrifugation (5000 rpm, 15 min, 4°C), washed twice with phosphate buffer saline (PBS) and resuspended in phosphate buffer (0.1 M, pH 7). Binding assay mixture comprised of 800 µl phosphate buffer, cell suspension (OD_{600} = 1, 10^9 cfu/ml) and 100 µl of acridine orange at a final concentration of 50 µg/ml. The assay mixture was incubated on shaker (90 rpm, 37°C) for 30 min, centrifuged (5000 rpm, 10 min, 4°C), and the supernatant collected was scanned (200-700 nm) using UV-Visible

spectrophotometer (UV1601, Shimadzu, Japan) and also analyzed for HPTLC analysis. Positive control consisted of phosphate buffer, and acridine orange but not the cells; negative control contained cell suspension without acridine orange. The acridine orange binding was analysed using HP-TLC (Camag Linomat 5). 10 µl sample was applied using auto-sampler to precoated plates (Silica gel 60 F_{254}: Merck, Darmstadt, Germany), developed with mobile phase consisting of acetonitrile: water (60: 40), and scanned at 494 nm.

Factors influencing the binding of AO by Lactobacillus strains

Binding assay was performed as described above varying (i) incubation time (0-180 min), (ii) pH (2-10) (iii) cell density (OD_{600} = 0.1, 0.5, 1 and 2), and (iv) AO (10-100 µg/ml). The assay mixture was prepared using 100 mM glycine-HCl (pH 2, 3), acetate (pH 4, 5), phosphate (pH 6, 7), tris (pH 8, 9) and glycine-NaOH (pH 10) buffers. The supernatant obtained upon centrifugation was analysed for residual acridine orange by UV-Visible spectrophotometry.

Pretreatment of lactobacilli

Cells were pelleted by centrifugation (5000 rpm, 15 min, 4°C) from 24 h *Lactobacillus* cultures growing in MRS medium at 37°C, and washed twice with PBS. The cells were then subjected to various treatments by suspending them in phosphate buffer (control-untreated), heat-killed (100°C, 15 and 30 min), 2 M HCl, 2 M NaOH, 2 and 8 M urea, 0.013 M β-mercaptoethanol, 0.1 M SDS, $MgCl_2$, $CaCl_2$, and 0.61 M TCA, and incubated at 37°C for 1 h. The cells suspended in 5 M LiCl and 50 mM $NalO_4$ were incubated at 4°C for 1 and 24 h respectively. One drop of ethylene glycol was added to cells suspended in sodium metaperiodate, to destroy the excess of metaperiodate present in the reaction mixture as described by Sreekumar and Hosono (1998b). The cells pelleted by centrifugation after the treatments, were washed twice with PBS and resuspended in phosphate buffer. These pretreated cells were used in the binding assays as described above. The cell density of all the pretreated cells was adjusted to OD_{600} = 1 and used for binding with acridine orange.

Binding of AO with surface-associated proteins and cell extract

Surface-associated proteins were extracted from cells as described by Sanchez *et al*. (2009). Cells pellet was suspended in 10 ml of 5 M LiCl, vortexed and incubated on shaker (80 rpm) for 30 min at 37°C. The cells were removed by centrifugation (10000

rpm, 20 min, 4°C), and the supernatant was filtered through 0.22 µm filter (Millipore) and evaluated for its binding abilities as described above. Intracellular cell extract was prepared by sonication. Cell suspension (10^9 cfu/ml) in phosphate buffer was sonicated (60 amplitude x 2 min, at 4°C) using Ultraschall Homogenisator Lab Sonic (Probe ϕ 2 mm x 80 mm, Sartorius, Labsonic M, Germany) for 5-6 cycles and centrifuged (10,000 rpm, 25 min, 4°C). Supernatant was filtered through 0.22 µm filter (Millipore) and used as cell extract for binding assay.

Binding of AO with simulated gastro-intestinal fluid treated cells

The SGF- and SGF-SIF-treated cells prepared as previously described (Pithva *et al*., 2014) were used in the binding assay with AO.

Statistical analysis

The values given here are the mean of two independent experiments ± standard deviation. The data were analyzed by one-way analysis of variance (ANOVA) using Microsoft Excel 2010. *P* values of <0.05 were considered significant.

Results

Binding of Acridine orange

Non-growing viable cells of *Lactobacillus* strains instantaneously bound AO but to varying extent (24-33 µg) within 1 min of incubation (Fig. 1 a & b). Thereafter, the amount of AO bound increased albeit negligible before becoming steady with no further adsorption occurring even upon extended incubation for 3-24 h. The complex between cells and AO was stable irrespective of the incubation time. HP-TLC chromatogram showed complete disappearance of AO spot in the binding assay mixtures incubated for 120 min and no new spot was evident in the treated samples (Fig. 1c). AO adsorption by the bacterial cells was pH-dependent; the cells were incubated for 3 h to provide sufficient time for AO binding. Strains Fb and 231 bound AO poorly at pH 2-5, and showed increased binding in the pH range 5-7 (Fig. 2). The amount of AO bound by the *Lactobacillus* strains GG, Vc and Actimel increased with increase in the pH 2-7 before becoming steady. Binding of AO (50 µg) with varying cell biomass exhibited strong correlation (r=0.86) between cell density and AO bound (Fig. 3). The strains showed differences in AO binding at lower cell densities ($OD_{600}\leq$ 0.5), but no significant difference ($P>0.05$) was observed among the strains at higher cell densities ($OD_{600}\geq$ 1).

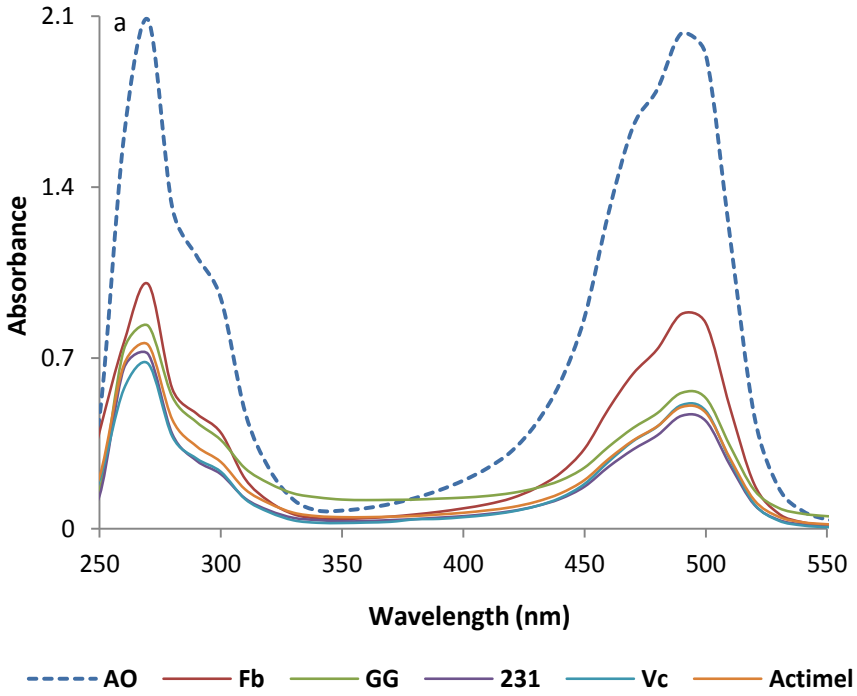

Fig. 1a UV-Visible spectra of residual supernatant of Acridine orange and co-incubated with the cells of *Lactobacillus* strains

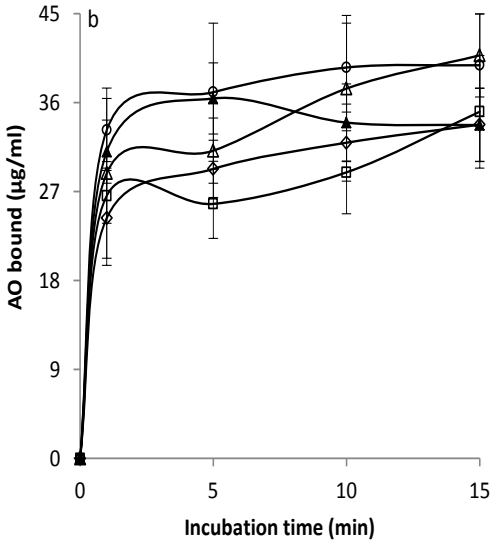

Fig. 1b Influence of incubation time on adsorption of AO by *Lactobacillus* strains *L. rhamnosus* Fb (◊), GG (□), 231(Δ), Vc (▲) and Actimel (○). Error bars indicate standard deviation

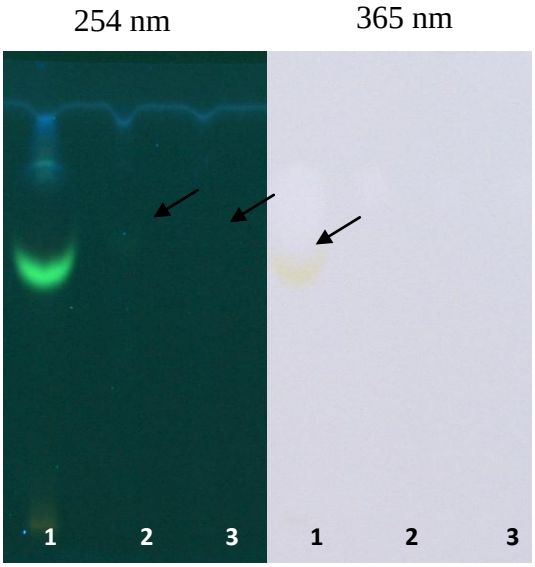

Fig. 1c HP-TLC chromatogram of residual supernatant of Acridine orange (AO) and incubated with bacterial cells, lane 1. control AO, lane 2. cells+AO (30 min) and lane 3. Cells+AO (120 min)

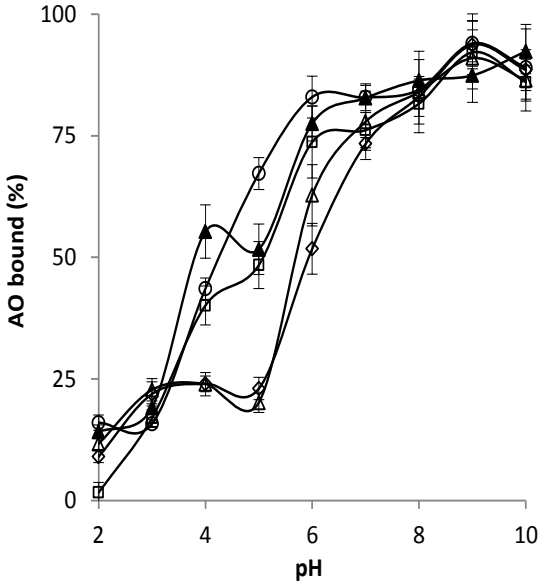

Fig. 2 Effect of pH on the binding of AO to the cells of *Lactobacillus rhamnosus* Fb (◊), GG (□), 231 (Δ), Vc (▲) and *L. casei* Actimel (○). Error bars indicate standard deviation

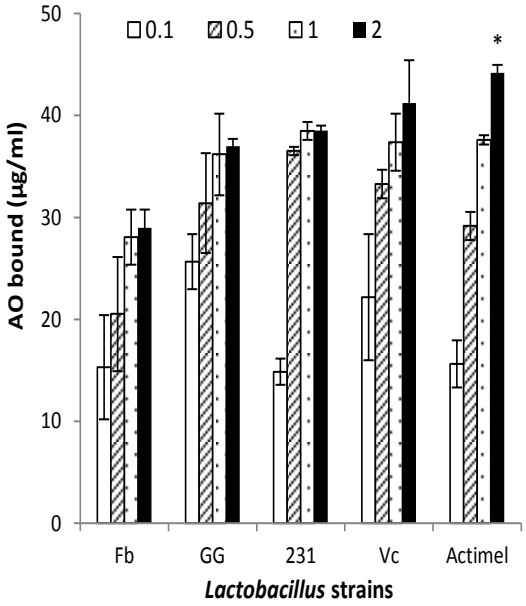

Fig. 3 Effect of various cell densities (OD_{600} = 0-2.0) of *Lactobacillus* strains Fb, GG, 231, Vc and Actimel on the binding of AO. Binding v/s cell densities was analysed at a significance level of $P<0.05$

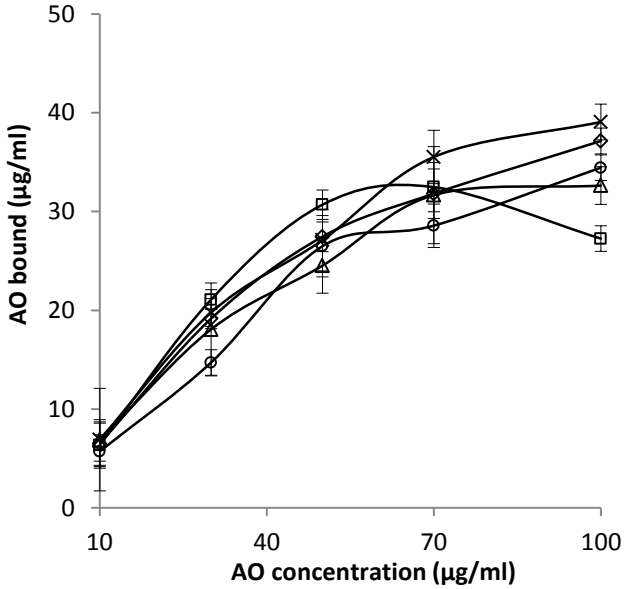

Fig. 4 Influence of AO concentration (10-100 µg) on the AO binding by *Lactobacillus* strains *Lactobacillus rhamnosus* Fb (◊), GG (□), 231 (Δ), Vc (×) and *L. casei* Actimel (○). Error bars indicate standard deviation

The amount of AO bound by the cells increased with AO concentration till 70 µg, except in GG (Fig. 4). At AO concentration beyond 70 µg, no further increase occurred in the bound AO. Cell debris did not significantly; ($P< 0.05$) bound AO. The amount of AO bound was extracted more in the first wash than the second wash. The complexes formed between AO and tested strains at 100 µg/ml AO were significantly more stable than those formed at <100 µg/ml. Heat-killed and viable cells exhibited similar retention abilities (Table 1).

Table 1. Extractability of AO bound to the cells of *Lactobacillus* strains

Strains	AO bound or released (µg/ml)							
	viable cells				heat-killed cells			
	Initial	1st wash	2nd wash	DMSO	Initial	1st wash	2nd wash	DMSO
	5 min (10 µg/ml)							
Fb	3.8±1.9	2.0±0.4	1.1±1.8	0.5±1.1	3.7±0.4	2.2±1.9	0.7±1.1	1.9±1.5
GG	4.8±3.7	2.4±1.0	1.0±0.4	1.3±0.4	4.5±1.6	2.4±1.7	0.6±5.5	4.2±0.2
231	4.9±2.0	2.2±3.9	1.8±1.1	2.0±2.2	3.5±1.0	2.9±2.3	0.7±3.1	2.6±0.8
Vc	4.4±1.1	1.6±1.8	0.9±0.2	2.3±4.8	3.6±2.9	2.9±1.8	1.2±1.4	2.1±2.9
Actimel	3.9±0.5	2.1±2.3	1.4±1.5	1.4±3.1	3.1±0.3	1.7±4.1	1.0±2.5	2.3±0.4
	180 min (10 µg/ml)							
Fb	6.3±2.1	2.2±2.8	2.2±1.7	0.9±1.2	7.2±0.6	4.4±3.5	1.9±1.1	2.4±1.9
GG	7.3±4.3	2.2±3.1	2.0±0.4	1.2±0.7	7.4±1.8	4.4±0.2	2.3±2.9	1.0±0.6
231	7.5±5.6	1.9±4.1	1.0±1.5	1.4±3.2	7.8±3.3	1.8±1.4	1.3±0.8	3.4±1.1
Vc	7.1±1.8	2.4±2.3	1.6±4.8	2.0±1.7	7.6±2.1	1.3±1.6	1.0±1.5	1.4±3.8
Actimel	7.6±2.4	1.8±1.6	1.1±1.9	6.3±0.5	7.8±5.5	1.9±3.3	1.5±1.4	3.9±4.3
	180 min (100 µg/ml)							
Fb	85.9±6.3	5.3±1.0	4.1±1.9	11.0±3.3	81.2±0.3	4.9±0.8	4.1±3.2	43.2±1.4
GG	89.6±1.7	10.7±8.1	4.1±2.3	21.6±2.9	85.2±4.2	7.6±1.4	4.8±1.9	45.4±1.8
231	84.3±3.1	3.8±0.6	4.3±0.5	47.6±4.1	79.5±1.8	4.7±0.8	4.6±1.7	42.5±2.0
Vc	78.5±1.4	2.9±1.7	4.6±0.3	30.0±6.5	75.2±0.6	8.2±4.4	2.8±0.4	47.8±3.3
Actimel	85.0±4.5	3.9±2.8	5.6±3.3	37.2±1.8	86.2±2.1	6.8±4.3	4.0±1.4	31.6±8.7

Fb – *Lactobacillus rhamnosus* Fb; GG – *Lactobacillus rhamnosus* GG; 231 – *Lactobacillus rhamnosus* 231; Vc – *Lactobacillus rhamnosus* Vc; and Actimel – *Lactobacillus casei* Actimel

Pretreatment of lactobacilli

The amount of AO bound by heat-killed and viable cells was similar. HCl, NaOH, $MgCl_2$ and $CaCl_2$ treatments caused reduction in the amount of AO bound. NalO4 and urea treatments did not reduce the binding ability of the tested strains on the contrary SDS, LiCl, TCA, and β-mercaptoethanol treatment were observed to increase the binding of AO (Fig. 5a). The effect of various treatments on AO adsorption is similar in both the strains Fb and GG. The treatments that reduced AO binding, exhibited concentration-dependent gradient effect when cells were incubated with varying concentrations of (0.1 to 2 M) HCl or NaOH; i.e., amount of AO bound decreased. Similar effect was also observed with the divalent cations (Fig. 5b)

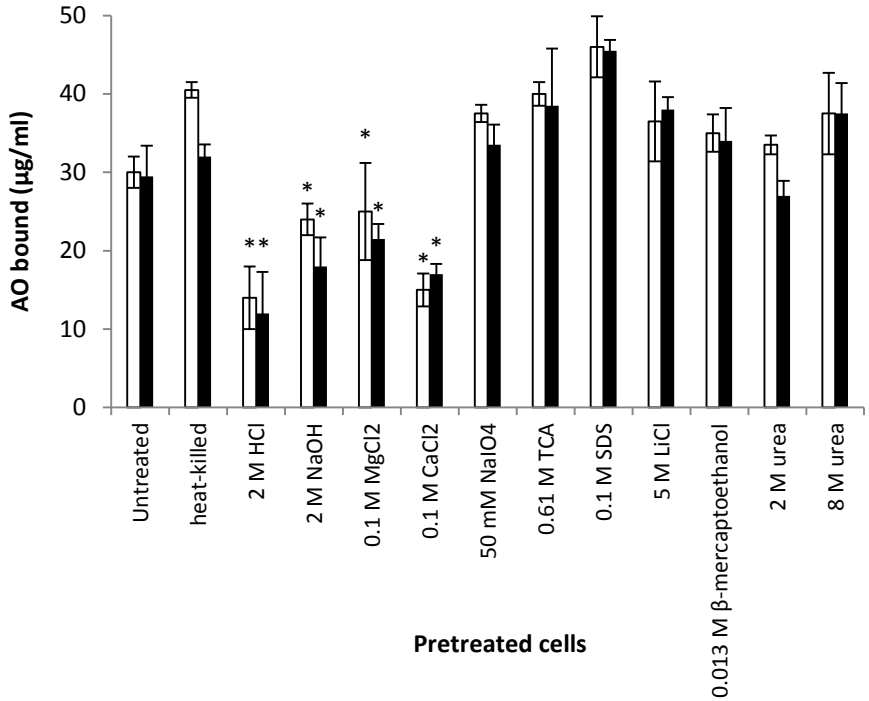

Fig. 5a Binding efficiencies of pretreated cells of *Lactobacillus rhamnosus* Fb (□) and GG (■) with AO. *indicates significantly lower AO binding (P<0.05), error bars indicate standard deviation

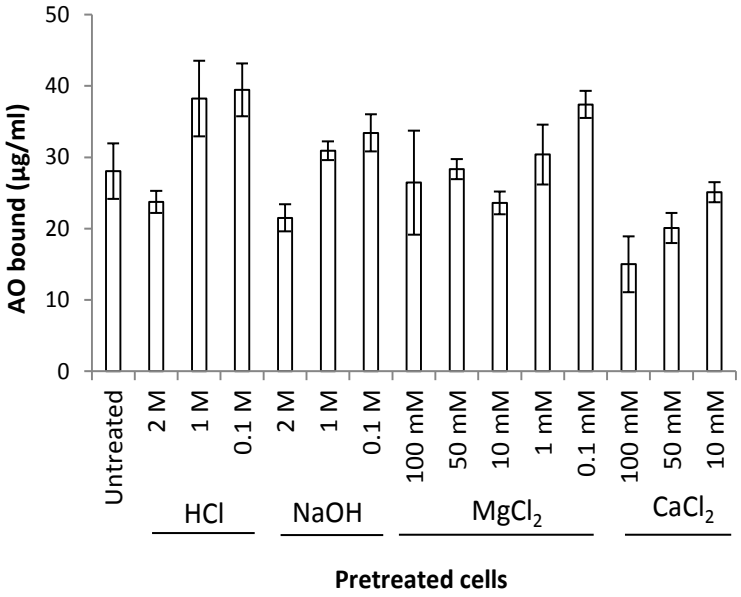

Fig. 5b Effect of HCl, NaOH, $MgCl_2$ and $CaCl_2$ concentration on AO binding by *L. rhmanosus* Fb, error bars indicate standard deviation

Binding of AO with simulated gastro-intestinal fluid treated cells

Cells treated with simulated gastro-intestinal fluid demonstrated AO adsorption abilities similar to that of untreated cells (Fig. 6).

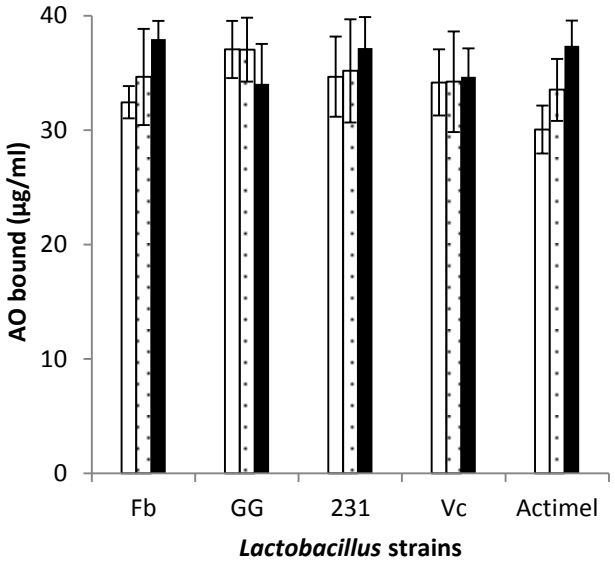

Fig. 6 AO bound by untreated cells (□), SGF-treated cells (⧈), and SGF-SIF-treated cells (■) of *Lactobacillus* strains. Values are represented as mean of three replicates and error bars show standard deviation

Discussion

The present study provides experimental evidences of the ability of human origin *Lactobacillus rhamnosus* strains to bind acridine orange, an intercalating agent responsible for frame shift mutation. Genotoxic substances may be either environmental xenobiotic or endogenously formed or food-borne, and may induce genetic alteration thereby leading to mutation and carcinogenesis (Wogan *et al*., 2004). Food contaminants and toxicants entering body through the oral route are exposed to the action of gut microbiota. Under such conditions, lactic acid bacteria present in gastrointestinal tract can serve as antimutagenic agents by decreasing the bioavailability of the toxicant through binding, and thereby decreasing the exposure to intestinal epithelium.

AO adsorption by *Lactobacillus rhamnosus* strains was evidenced from the UV-Visible spectrum and HP-TLC analysis where no evident appearance respectively of new peak and new spot observed in the samples incubated with cells. Moreover, AO adsorption occur instantaneously with only marginal increase in the amount of AO bound during prolonged incubation for 24 h. 24-34 µg AO is adsorbed within 1 min of addition of cells. Ambalam *et al*. (2011) has also reported instantaneous AO binding by *L. rhamnosus* 231. AO adsorption ability of isolated strains Fb and Vc is comparable to that of the reference strains GG, 231 and Actimel. AO adsorption is attributed to the extracellular cell wall components. *In vitro,* binding and antimutagenic activity of yeast cell wall mannans against AO has been attributed to its absorptive capability (Krizkova *et al*., 2001). Binding is one of the antimutagenetic processes, as the toxicants bound to the cells are not available to adsorb colon epithelium. Antimutagenic activity of LAB against heterocyclic amines involves binding which in turn limits adsorption to the gut epithelium (Orrahage *et al*., 1994).

pH exerts marked influenced on the AO binding capacity of *Lactobacillus* strains. *Lactobacillus* strains can be divided in the two groups on the basis of their AO binding capacity; (i) strain Fb and 231 bind AO in narrow pH range 7-10 and poorly in pH range 2-5, (ii) Strains GG, Vc and Actimel bind AO optimally in the pH range 6-10, this AO binding decreases with pH. These observations allow us to hypothesize, that in the intestine where the absorption of food is maximum and pH is around 7 can favor the binding of AO by *Lactobacillus* cells. In fact, the cells can bind AO throughout the GIT

but to a varying extent determined by the pH. Simulated gastrointestinal fluid treated cells did not lose the AO binding ability indicating further that the probiotic strains can indeed play an important role in the removal of AO. Sreekumar and Hosono (1998) have reported pH-dependent binding of Trp-P-1 by *L. gasseri* and *B. longum* cells with optimum binding at pH 7. The binding of AO by the cells is also dependent on cell density and AO concentration.

Chemical and physical treatments aimed at inactivating the specific components of the bacterial cell wall did not totally abolish AO binding. Heat-killed cells bind AO as efficiently as viable cells and therefore can also contribute in the mutagen removal. Heat treatment does not reduced binding ability of the cells implying that the mutagen is just bound and not metabolized by the cells. Heat-treated lactic acid bacteria have previously been shown to effectively bind amino acid pyrolysates (Orrahage *et al*., 1994) and aflatoxins (Thyagaraja and Hosono 1994). Niderkorn *et al.* (2006) reported heat-treated non-viable cells bound higher zearlone and fumonisins than viable cells. In addition to this, AO adsorption occurs only by intact bacterial cells as evidenced from the inability of surface layer protein extracts and cell extracts to bind AO. Moreover, destruction of bacterial cells strongly inhibited AO binding, indicating the importance of overall outer cell wall architecture for the AO binding by the cells. Treatments such as SDS, LiCl, TCA, and β-mercaptoethanol that cause denaturation of proteins were observed to increase AO binding clearly indicates proteins are not responsible for AO binding. $NaIO_4$ that cause oxidation of polysaccharides does not affect AO binding, excluding the implication of cell surface polysaccharides in AO binding. Urea treatment did not reduce the binding indicates that hydrophilic interactions are involved in the binding mechanism, since urea is an anti-hydrophobic agent and breaks hydrogen bonds. This also suggests that hydrogen bond formation is not important for the binding of AO by *Lactobacillus* cells.

The abolition of specific components of the bacterial cell wall, e.g. carbohydrates and teichoic acids resulted in reduction in AO binding by cells, implicating the importance of these components for AO binding. Acid and alkali treatments are known to extract the carbohydrates and proteins from the cells. Divalent cations, calcium (Ca^{2+}) and magnesium (Mg^{2+}) implicates importance of ionic interactions and participation of acidic centers in the cell wall such as teichoic and lipoteichoic acids, in AO binding. Similar inhibition of aflatoxin binding by *Lactobacillus* GG in the presence of Ca^{2+} ions

has been reported (Haskard *et al*., 2000). The amount of AO bound by variously treated cells is less still substantial amount of AO is bound which suggest involvement of multiple components in AO binding and therefore different mechanisms. Graded response exerted by varying concentration of HCl, NaOH and divalent cations substantiate the involvement of carbohydrates, proteins and teichoic acids in AO binding. AO binding is observed to be partially reversible as upon aqueous extraction 20-30% AO gets released. Moreover, the complex formed between the cells and AO is stable regardless of the cell viability, and incubation time but it depends on the AO concentration.

Overall, the experimental evidences presented indicate that the binding of AO occurs mainly to the cell wall components such as carbohydrates, proteins and teichoic acids and involves hydrophilic interactions. Heat-killed cells bind AO similar to the viable cells implies that non-viable cells are also useful for the removal of AO. Probiotic strains with binding abilities could inactivate harmful toxicants, which alter their mutagenic and carcinogenic potential. Further studies are being undertaken to address the antimutagenic activity of *Lactobacillus* strains against AO. Such probiotic strains can also be used as a decontamination method for the removal of toxicants from the GIT.