5 Biotransformation and Detoxification of 4-nitroquinoline-1-oxide by *Lactobacillus rhamnosus* Strains

Introduction

Mutagens and carcinogens occurring in food and environment are of great concern due to their implications on human health (Roy *et al.*, 2002). The complete elimination of such toxicants from the human body seems to be impossible due to their ubiquitous presence. Therefore, alternative approaches have been proposed as protective strategies, namely, (i) deliberate consumption of antimutagens that may inactivate or reverse the effects of some classes of mutagens, (ii) avoiding the exposure to recognized risk factors, and (iii) modulating defense mechanism of host (Ferguson, 1994). One of the possible ways of reducing the incidence of cancer in human is deliberately increasing the antimutagen level. Antimutagens are of two types, desmutagens which cause extracellular modification of mutagens (thereby preventing DNA damage), and bioantimutagens, which decrease the mutation rate by virtue of their involvement in cellular processes (Kada *et al.*, 1986). In particular, antigenotoxicity is now frequently included among the functional properties characterizing the probiotic bacteria (Burns and Rowland, 2000). To a considerable extent, such antimutagens are presented by lactic acid bacteria and propionic acid bacteria. The most promising aspect is exploring the natural inhabitants of the gastrointestinal tract e.g. lactobacilli as antimutagens, as they are not only non-pathogenic to humans, but they also possess health-promoting properties. Anticarcinogenic action of probiotic lactic acid bacteria is manifested by multifactorial mechanisms: maintenance of gut microflora homeostatis, inhibition of colonic enzyme activity, inhibition of other potentially harmful bacteria, interaction with potentially harmful bacteria, interaction with colonocytes, stimulation of the immune system, production of physiologically active metabolites (eg. SCFA), and binding or degradation of carcinogens and toxins (Burns and Rowland, 2004). Many studies have reported binding and antigenotoxic activity of probiotic lactobacilli and Bifidobacteria bacteria against procarcinogens, *viz.* (i) food-borne mutagens: polycyclic aromatic hydrocarbons, heterocyclic aromatic amines, protein pyrolysates,

nitrosamines, furfurols, (ii) mycotoxins (aflatoxin and ochratoxin), and (iii) other chemical mutagens such as alkylating agents, nitroarenes, antimicrobial agents, etc. (Haskard *et al*., 2001; Ambalam *et al*., 2011). Antigenotoxic activity has been reported mainly for probiotic strains (*Lactobacillus* and Bifidobacteria) isolated from dairy products and fermented foods (Lankaputhra and Shah, 1998; Cenci *et al*., 2002; Caldini *et al*., 2005; Corsetti *et al.,* 2008).

Lactobacillus rhamnosus Fb and Vc strains used in the present study exhibit various 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, and (vi) susceptible to a range of antibiotics making the strains safer for their use as probiotics (Pithva *et al*., 2014). The aim of the present study was to evaluate antimutagenic and antigenotoxic activities of *Lactobacillus rhamnosus* strains against 4-nitroquinoline-1-oxide (nitroarene), a direct acting mutagen causing strand scission and formation of DNA adduct. Antimutagenic activity was evaluated by Ames test, the known point reverse mutation assay on *Salmonella typhimurium* (Ames *et al*., 1975; Mortelmans and Zeiger, 2000). Genotoxicity was evaluated employing SOS-Chromotest, short-term bacterial assay to detect the DNA damaging agents by monitoring the SOS response in *Escherichia coli* PQ37 (Quillardet and Hofnung, 1985). Additionally, the antimutagenic and antigenotoxic activities of *Lactobacillus* strains were compared with well-established probiotic strains – *L. rhamnosus* GG (ATCC 53103), *L. rhamnosus* 231 (Ambalam *et al*., 2014) and *L. casei* DN 114 001 (Actimel strain, DANONE, France).

Materials and Methods

Bacterial strains and culture conditions

Lactobacillus strains 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 used were *L. rhamnosus* GG (ATCC 53103), *L. rhamnosus* 231 (EF661653) and *L. casei* DN 114 001 (Actimel strain, DANONE, France). *Lactobacillus* strains were grown in de Man-Rogosa-

Sharpe (MRS; Himedia, Mumbai, India) for 24 h at 37°C. The stock cultures were maintained in 10% (wt/vol) skim milk at 4°C.

Salmonella typhimurium (*his*$^-$) TA-98 used for mutagenicity was a kind gift from Dr. Ramadasan Kuttan, (Amala Cancer Research Center, Kerala, India). It was grown in Nutrient broth II (Himedia, India) for 18 h at 37°C. *Escherichia coli* PQ37 (*sfiA::lacZ*) procured from Institute Pasteur (Paris, France) for genotoxicity assay, was grown in Luria broth (Himedia, India) for 12-15 h at 37°C.

Chemicals

4-nitroquinoline-1-oxide (4-NQO) used in the study was obtained from Lancaster (Alfa Aesar, CAS no. 56-57-5). Stock solution of NQO (1 mg/ml) was prepared in dimethyl sulphoxide (Merck) and stored at 4°C. Working solution (0.1 mg/ml) was prepared just before testing. *o*-nitrophenyl-β-D-galactopyranoside and *p*-nitrophenyl phosphate were obtained from Sigma.

Co-incubation assay with 4-nitroquinoline-1-oxide

Twenty four old cells of lactobacilli grown in MRS broth at 37°C were pelleted by centrifugation (10000 rpm, 15 min, 4°C), washed twice with phosphate buffer saline (PBS – 0.1 M, pH 7.2) containing 0.85% (wt/vol) NaCl and resuspended in phosphate buffer (0.1 M, pH 7.0). One ml of co-incubation assay mixture comprising of 800 µl phosphate buffer, 4-NQO at final concentration of 53 µM and cell suspension (OD_{600} = 1.0, 10^9 cfu/ml) was incubated on shaker (90 rpm, 37°C) for 180 min, centrifuged (5000 rpm, 10 min, 4°C), and the supernatant collected was filter sterilized using 0.22 µm filter (Millipore) and scanned (200-400 nm) using UV-Visible spectrophotometer (UV1601, Shimadzu, Japan). Co-incubation was performed with varying incubation time 30-180 min and heat-killed cells (100°C for 15 and 30 min). The residual genotoxicity and mutagenicity in the supernatant was evaluated by SOS-Chromotest and Ames test respectively. Appropriate controls were also included in the study. Pelleted cells were then washed twice with PBS and appropriate dilutions in PBS were plated on MRS agar by pour plate method. Viability (%) was calculated in comparison to the control (lactobacilli without 4-NQO). If not indicated otherwise, the co-incubation was performed as described above.

Influence of cell density, pH and 4-NQO concentration on biotransformation of 4-NQO

Co-incubation assay was performed varying (i) cell density (OD_{600} = 0.1, 0.5, 1 and 2), (ii) pH (2-10) and (iii) 4-NQO concentration (53-263 µM). The supernatant was analysed as described above. Co-incubation 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.

Simulated gastrointestinal fluid treated cells

The SGF- and SGF-SIF-treated cells prepared as previously described by Pithva *et al*. (2014), were used in the co-incubation assay with 4-NQO.

Influence of pretreated lactobacilli cells on 4-NQO biotransformation

Twenty four hour old lactobacilli cells grown in MRS medium at 37°C were pelleted by centrifugation (10000 rpm, 15 min, 4°C), and washed twice with PBS. The cells were subjected to various treatment by by suspending them in phosphate buffer (control-untreated), (2 M) HCl, (2 M) NaOH, (2 M) and (8 M) urea, (0.013 M) β-mercaptoethanol, (0.1 M) SDS, (0.1 M) $MgCl_2$, (0.1 M) $CaCl_2$, and (0.61 M) TCA, and incubated at 37°C for 1 h. The cells suspended in (5 M) LiCl and (50 mM) $NaIO_4$ were incubated at 4°C for 1 and 24 h respectively. One drop of ethylene glycol was added to the cells suspended in $NaIO_4$; to destroy excess metaperiodate present in the reaction mixture as previously described (Sreekumar and Hosono, 1998). The cells were pelleted by centrifugation, were washed twice with PBS and resuspended in phosphate buffer. These pretreated cells were used in the co-incubation assay and for the determination of cell viability.

Preparation of surface-associated protein extracts

Surface-associated proteins were extracted from cells as previously described by Sanchez *et al*. (2009). Cell 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 (Millipore) used as surface-associated protein extracts.

Preparation of intracellular cell extracts

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 (10000 rpm, 25 min, 4°C). Supernatant was filtered through 0.22 µm filter (Millipore) and used as cell extract.

Co-incubation assay

The co-incubation assay was performed using cell extracts or surface associated protein extracts (900 µl) instead of cells suspension. Antigenotoxicity and antimutagenicity was evaluated as described below.

Genotoxicity assay: SOS-Chromotest

Some of the responses induced in *E. coli* PQ37 by DNA-damaging agents involve a set of functions known as the SOS responses as well as 'damage-inducible' genes. The assay is based on the activation of SOS-response in *E. coli* PQ37 strain (Quillardet and Hofnung, 1985) that carries *sfiA::lacZ* gene fusion and is *lac*$^{-}$. β-galactosidase activity is therefore strictly dependent on *sfiA* expression. The constitutive alkaline phosphatase production in PQ37 was used as an indicator of protein synthesis in the presence of genotoxins. The *uvrA* mutation renders the strain deficient in excision repair and accordingly increases the response to certain DNA-damaging agents. An *rfa* mutation renders the strain lipopolysaccharide deficient and allows better diffusion of certain chemicals into the cell. *E. coli* PQ37 culture (0.1 ml) grown overnight in Luria broth containing ampicillin (20 µg/ml) was transferred to 5 ml of the same medium and incubated on shaker (100 rpm, 37°C, 2 h). The OD_{600} of this culture was adjusted to 0.3-0.4 as required for the assay. One ml of this culture was added to 9.0 ml of fresh Luria broth without ampicillin and 600 µl of this suspension was mixed with 20 µl of sample containing 4-NQO or 4-NQO treated with cells/cell extracts/surface associated protein extracts and incubated for 2 h (100 rpm, 37°C). β-galactosidase and alkaline phosphatase activities were evaluated colorimetrically at 420 nm using *o*-nitrophenly-β-D-galactopyranoside and *p*-nitrophenyl phosphate respectively. Positive and negative controls were prepared in phosphate buffer with or without 4-NQO, respectively. SOS induction factor (IF_{SOS}) = [(β-galactosidase/alkaline phosphatase-test)/ [(β-galactosidase/alkaline phosphatase-uninduced culture)] was determined. Enzyme activities are expressed as conventional units (U) = A_{420} x 1000/t, where t is the substrate conversion time in minutes.

Mutagenicity assay: Ames test

Mutagenicity was estimated by measuring the extent of reverse mutation of *Salmonella typhimurium* (*his⁻*) TA 98 auxotroph to prototroph (Ames *et al*., 1975; Mortelmans and Zeiger, 2000). Briefly, 100 µl of an overnight grown culture of TA 98 in Nutrient broth No. II (Himedia, India) was added to 0.5 ml of phosphate buffer (0.1 M, pH 7.4) containing 25 µl mutagen (4-NQO, or 4-NQO treated with cells/cell extract/surface associated proteins) sample to be tested. The mixture was pre-incubated at 37°C for 20 min, mixed with 2 ml of soft agar containing 0.05 mM histidine/biotin and poured on minimal glucose agar plates. Revertant colonies were counted after incubation of 48 h at 37°C. Positive control was also included in the experiment. Antimutagenic activity (%) was calculated using the formula (C_R-T_R/C_R) x 100, where C_R is number of revertant colonies in control, T_R is number of revertant colonies in treated.

HPLC analysis of 4-NQO

The supernatant obtained upon co-incubation of 4-NQO with or without cells was filtered through 0.22 µm filter (Millipore), and analysed by HPLC (Water 2489) using C_{18} column (Waters, Sunfire C_{18} 5 µ 4.6 x 150 mm) with an isocratic mobile phase of water: acetonitrile: triethylamine: glacial acetic acid (740:260:1:1) at a flow rate of 1 ml/min. The absorbance was monitored at 368 nm using UV-Visible detector.

Statistical analysis

The values given here are the mean of three 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. The correlation coefficient between antigenotoxic activity and biotransformation rate was calculated.

Results

Biotransformation of 4-NQO by Lactobacillus strains

The cells obtained from 6, 12, 18, 24 and 30 h old cultures upon co-incubation with 53 µM 4-NQO led to UV spectral modification but to varying extent implying biotransformation of 4-NQO by *Lactobacillus* cells. The spectral modification was maximum with 12 and 24 old cells of lactobacilli (Table 1). Therefore, all the experiments were carried out using cells obtained from 24 h old cultures of lactobacilli. The spectral shift towards shorter wavelength was proportional to the

reduction in genotoxic and mutagenic activities. The extent of UV spectral modification and reduction in genotoxicity and mutagenicity of 4-NQO varied with the *Lactobacillus* strain used in the co-incubation assay. *L. rhamnosus* Fb, *L. casei* Actimel and *L. rhamnosus* 231 exhibited ≥95% antigenotoxic, and ≥74% antimutagenic activities (P<0.05), and was accompanied with bigger spectral shift towards shorter wavelength λ_{347}, $\lambda_{348,}$ λ_{351} respectively (Fig. 1 a & b). Moreover, antigenotoxic activity was observed to be proportional to the extent of UV spectral shift. The heat-killed cells did not induce modification in the UV spectrum of 4-NQO (Fig. 1 a), and did not reduce genotoxicity but reduced mutagenic activity by 20-30%. All the strains biotransformed 4-NQO, and reduced genotoxicity by 50% within 5 min of co-incubation. UV spectral shift and antigenotoxic activity significantly increased (P<0.05) with incubation time from 30 to 180 min (Fig. 1 c).

Table 1 Changes in the absorbance maxima during 3 h of co-incubation with 4-NQO (53 µM) with non-growing viable cells of *L. rhamnosus* Fb harvested at varying culture ages

Culture age (h)	λ_{max} of the co-incubation mixture (nm)
0	368
6	355
12	348
18	352
24	347
30	349

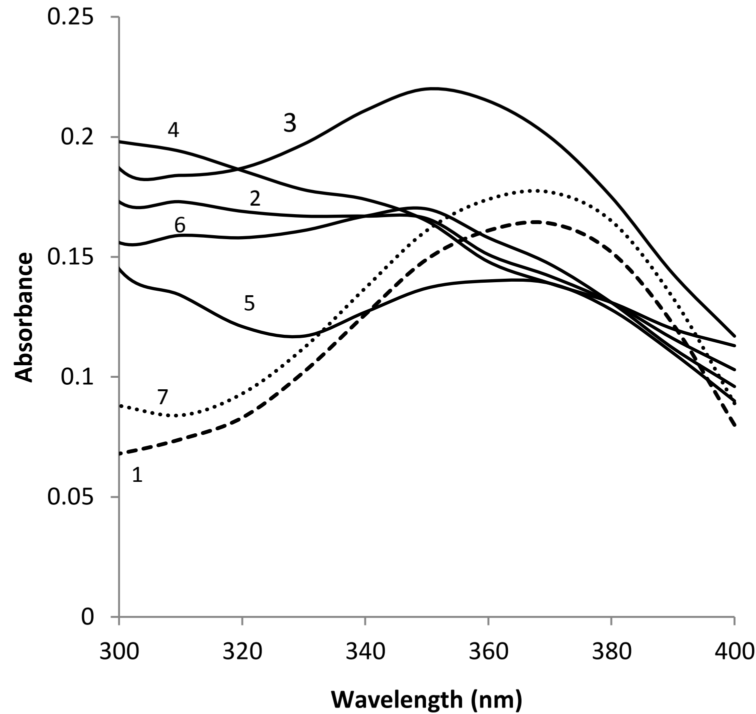

Fig. 1a UV spectral modification of 4-NQO after co-incubation with *Lactobacillus* strains 1. Control (without bacterial cells), λ_{368} (dashed line), 2. *L. rhamnosus* Fb λ_{347}, 3. *L. rhamnosus* GG λ_{351}, 4. *L. rhamnosus* 231 λ_{357}, 5. *L. rhamnosus* Vc λ_{364}, 6. *L. casei* Actimel λ_{348} and 7. Heat-killed cells λ_{368}

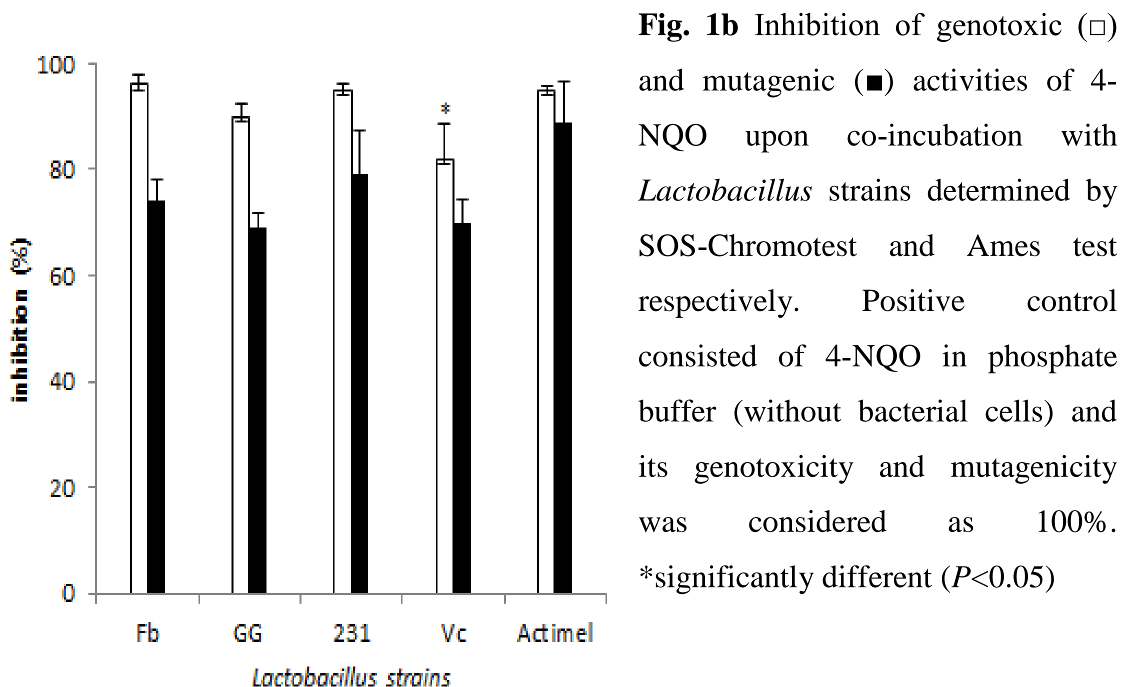

Fig. 1b Inhibition of genotoxic (□) and mutagenic (■) activities of 4-NQO upon co-incubation with *Lactobacillus* strains determined by SOS-Chromotest and Ames test respectively. Positive control consisted of 4-NQO in phosphate buffer (without bacterial cells) and its genotoxicity and mutagenicity was considered as 100%. *significantly different ($P<0.05$)

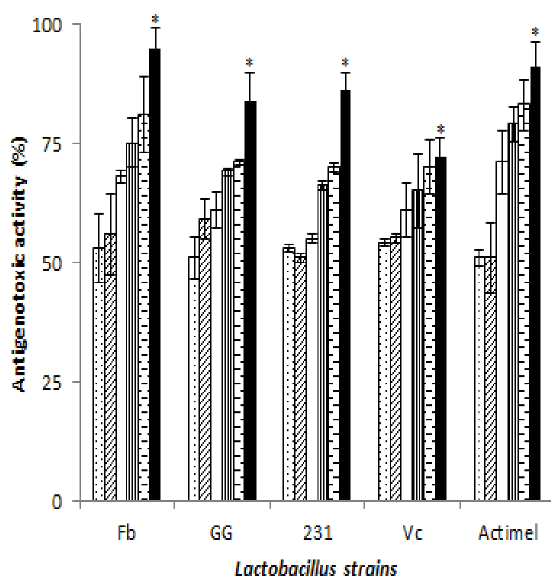

Fig. 1c Antigenotoxic activity of *Lactobacillus* strains against 4-NQO determined at different time interval of co-incubation with 4-NQO (▫) 5 min; (▨) 10 min; (□) 30 min (▥) 60 min; (⊟) 120 min and (■) 180 min by SOS-Chromotest. *indicates antigenotoxic activity significantly increased with incubation time

Influence of cell density, pH and 4-NQO concentration on biotransformation of 4-NQO

Biotransformation of 4-NQO was influenced by cell density, pH, and 4-NQO concentration during co-incubation assay. Biotransformation rates of 4-NQO and reduction in mutagenicity and genotoxicity showed high correlation (r=0.9) with cell density (Fig. 2 a). The shift in the λ_{max} was 3, 13, 18 and 19 nm respectively with the cell densities (OD_{600}) of 0.1, 0.5, 1 and 2. The maximum antigenotoxic and antimutagenic activities was observed with cell density of 1. Further increase in cell density increased antigenotoxic activity ($P<0.05$) with strains GG, 231, Vc and Actimel but not with the strain Fb (Fig. 2 b). Antimutagenic activity significantly increased with increase in cell density with strains Fb and GG, but the difference was not significant ($P<0.05$) with strains 231, Vc and Actimel. pH of the co-incubation mixture influenced the extent of 4-NQO spectral modification and antigenotoxic activity of lactobacilli (Table 2). The highest genotoxic reduction occurred at pH 7 with the strains Fb, 231 and Actimel, and at pH 8 with GG and Vc. The biotransformation rate of 4-NQO was lower in the pH range 2-5 and the antigenotoxic activity exhibited by the strains was <50%. Co-incubation of cells (10^9 CFU/ml) with 53-263 µM 4-NQO exhibited three trends in the 4-NQO biotransformation rate (Fig. 2 c). 4-NQO biotransormation rates (i) decreased at ≥211 µM 4-NQO in the case of Fb, (ii) became steady beyond 158 µM 4-NQO in the case of GG, 231 and Actimel, and (iii) increased but poorly with the increase of 4-NQO concentration in the case of Vc.

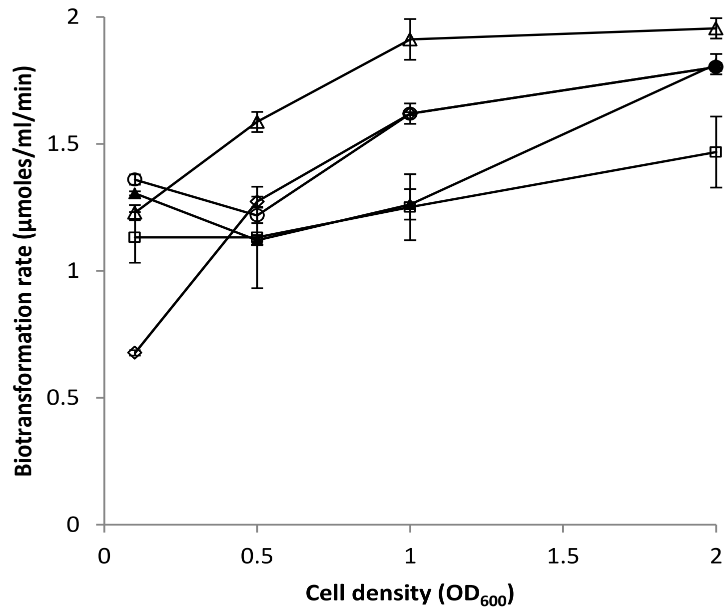

Fig. 2a 4-NQO biotransformation rates after 30 min of co-incubation with *Lactobacillus* strains (◊) *L. rhamnosus* Fb, (□) *L. rhamnosus* GG, (Δ) *L. rhamnosus* 231, (▲) *L. rhamnosus* Vc and (○) *L. casei* Actimel at different cell densities (OD_{600} = 0.1, 0.5, 1 and 2)

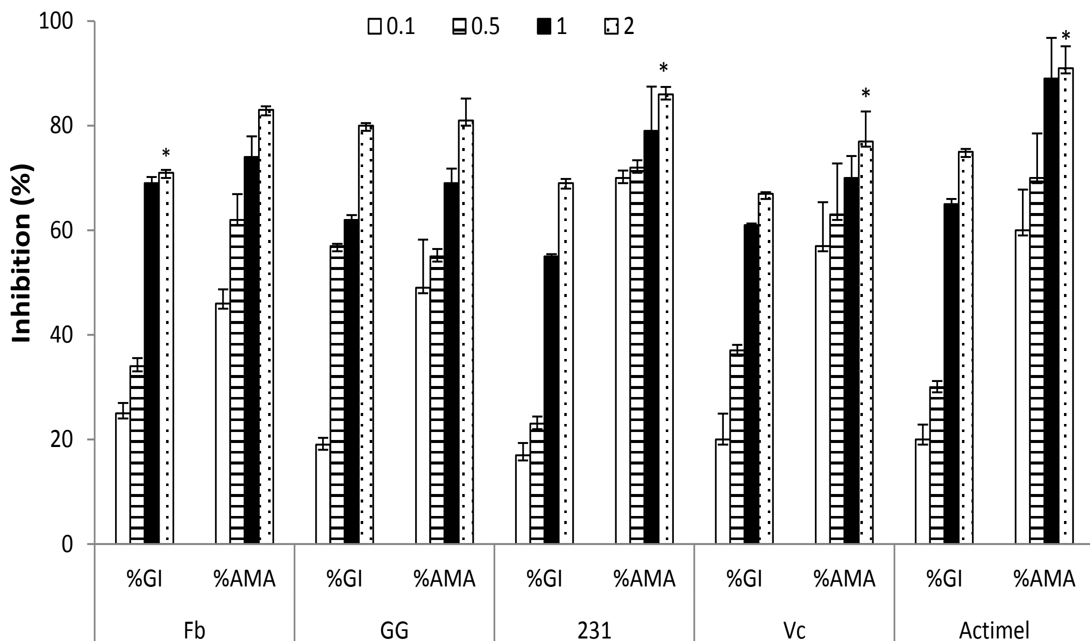

Fig. 2b Inhibition of genotoxic and mutagenic activities of 4-NQO by *Lactobacillus* strains at different cell densities (OD_{600} = 0.1 – 2) after 30 min of co-incubation determined by SOS-Chromotest and Ames test. *no significant difference (P<0.05)

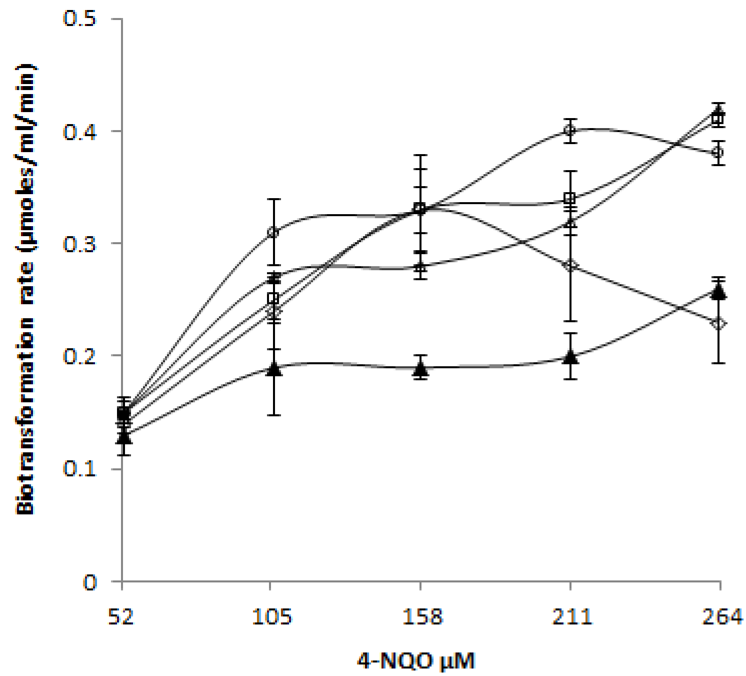

Fig. 2c Biotransformation rates of 4-NQO at 4-NQO concentrations of (53 - 263 µM) upon co-incubation with *Lactobacillus* strains (◊) *L. rhamnosus* Fb, (□) *L. rhamnosus* GG, (Δ) *L. rhamnosus* 231, (▲) *L. rhamnosus* Vc and (○) *L. casei* Actimel

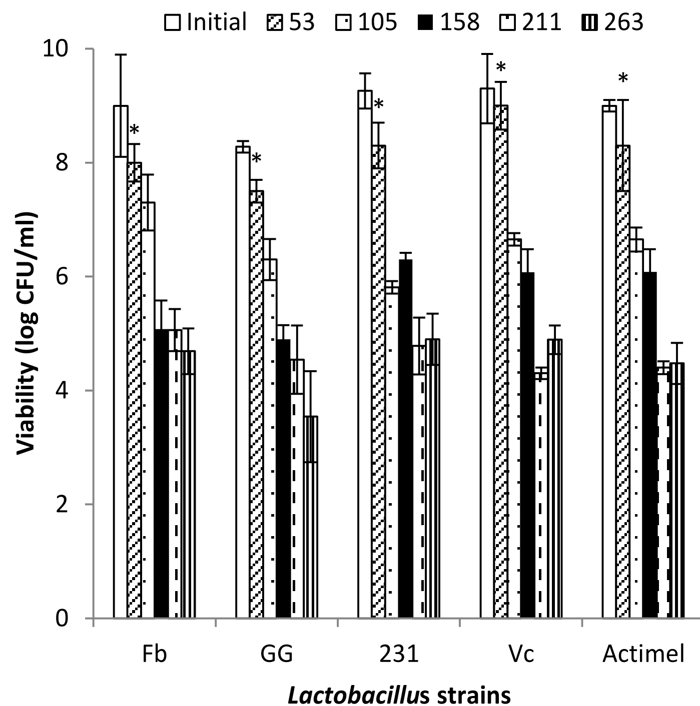

Fig. 2d Viability (log cfu/ml) of *Lactobacillus* strains after 3 h of incubation with 4-NQO concentrations (0-263 µM) determined by viable count method using MRS medium. *no significant difference in viability in comparison to initial count (P<0.05)

Antigenotoxic activity decreased significantly with the increase in the concentration of 4-NQO and was completely inhibited at 263 µM, but the strains 231 and Actimel retained 24 and 34% antigenotoxic activity, respectively (Table 2). The tested strains retained ≥89% viability in the presence of 53 µM 4-NQO after 3 h of co-incubation that reduced to 37-57% at 263 µM 4-NQO (Fig. 2 d).

Table 2. Influence of pH and 4-NQO concentration on genotoxicity inhibition by *Lactobacillus* strains as evaluated by SOS-Chromotest

	genotoxicity inhibition (%)±SD				
Parameters	Fb	GG	231	Vc	Actimel
pH					
2	45±0.02	37±0.35	38±0.01	43±0.02	38±0.04
3	49±0.19	47±0.16	33±0.19	52±0.03	49±0.02
4	46±0.20	28±0.19	38±0.36	39±0.51	49±0.91
5	46±0.01	28±0.11	38±0.04	39±0.10	49±0.10
6	79±0.11	74±0.33	75±0.40	69±0.01	84±0.80
7	92±0.40	83±0.01	85±0.01	70±0.10	93±0.20
8	90±0.01	86±0.21	77±0.01	80±0.60	78±0.12
9	81±0.21	68±0.03	74±0.02	73±0.12	79±0.50
10	88±0.10	67±0.05	78±0.05	75±0.04	68±0.41
4-NQO µM					
53	96±2.08	90±0.58	95±3.00	82±3.51	95±1.94
105	93±2.52	66±0.01	79±1.31	65±3.05	82±1.52
158	79±1.00	51±1.00	49±1.00	54±2.50	47±1.09
211	79±0.58	20±3.05	65±0.60	28±2.00	58±1.70
263	0±0.58	0±0.58	24±4.91	0±1.70	34±1.67

Simulated gastrointestinal fluid treated cells

The tested strains upon simulated gastric fluid transit demonstrated decreased 4-NQO biotransformation rates except Actimel. 4-NQO biotransformation abilities of SGF-SIF-treated cells were comparable to that of SGF-treated cells except with strains GG and 231 where it was found to decrease further (Fig. 3).

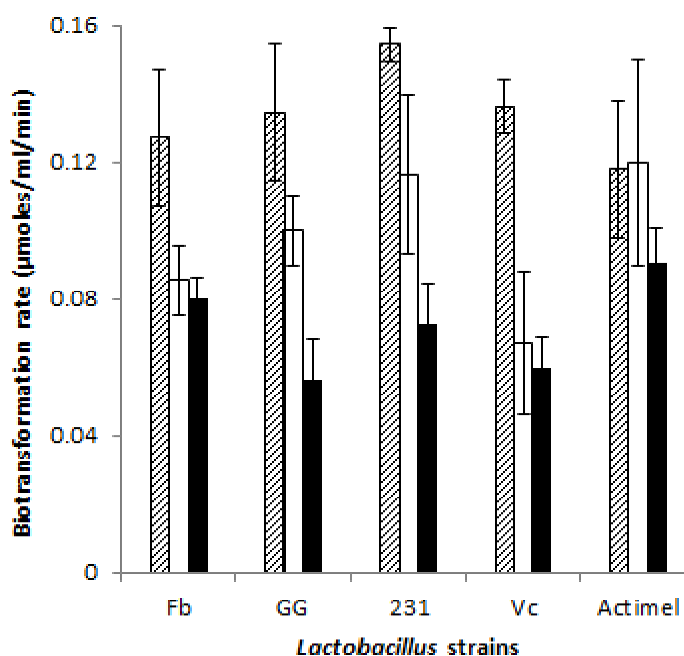

Fig. 3 Biotransformation rates of 4-NQO by untreated (▨), SGF-treated cells (□), and SGF-SIF-treated (■) cells of *Lactobacillus* strains

Influence of pretreated lactobacilli cells on 4-NQO biotransformation

Cells were subjected to various chemical treatments before co-incubation with 4-NQO in order to identify the cell wall component(s) involved in the binding and biotransformation of 4-NQO by lactobacilli cells. Co-incubation of 4-NQO with variously pretreated cells, exhibited reduced antigenotoxic and antimutagenic activities but to varying extent compared to untreated cells (Fig. 4). The HCl, NaOH, urea, β-mercaptoethanol, SDS, TCA and $NaIO_4$ treated cells did not induce marked spectral modification and exhibited significantly lower ($P<0.05$) antigenotoxic and antimutagenic activities. $MgCl_2$ and $CaCl_2$ treated cells showed 44-51% and 43-60% antigenotoxic and antimutagenic activities respectively. LiCl treated cells showed 44% antigenotoxic activity. Thin layer chromatography (data not shown) of the above co-incubation mixtures where antigenotoxicity >25% showed appearance of new spots indicating biotransformation of 4-NQO. Pretreatment of cells caused reduction in cell viability to varying extent. The viability of the cells treated with $CaCl_2$ and β-mercaptoethanol was >90%; and with $MgCl_2$ and LiCl was >80%, HCl, $NaIO_4$, NaOH, SDS and urea ≥74% and TCA was 65%.

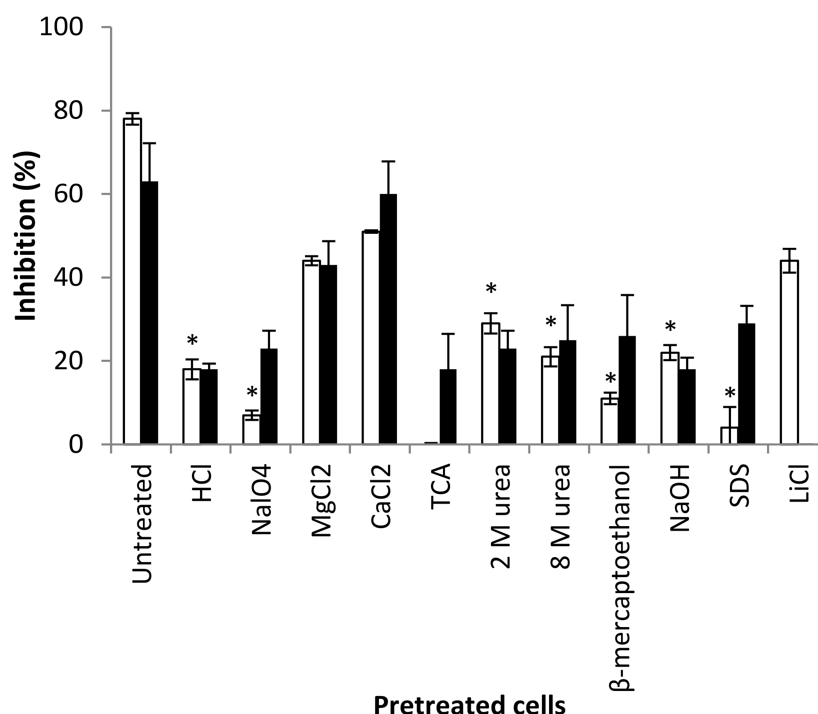

Fig. 4. Inhibition of genotoxic and mutagenic activities of 4-NQO after co-incubation with variously pretreated cells determined by SOS-Chromotest (□) and Ames test (■). The activities were determined after 60 min of co-incubation. *significantly decreased in comparison to untreated viable cells ($P<0.05$)

Antigenotoxic and antimutagenic activities of surface-associated proteins and cell extract against 4-NQO

Surface layer protein extracts and cell extracts neither caused UV spectral modification nor exhibited antigenotoxic and antimutagenic activities.

HPLC analysis

HPLC analysis provided additional evidence of 4-NQO biotransformation. The peak of the parent compound ($RT_{9.16\ min}$) disappeared with simultaneous appearance of new peak ($RT_{6.98\ min}$). Our inference that 4-NQO is not only biotransformed but extensively degraded evidenced from the size of peak $RT_{6.98\ min}$ decreased with the appearance of newer peaks in the samples co-incubated longer (Fig. 5).

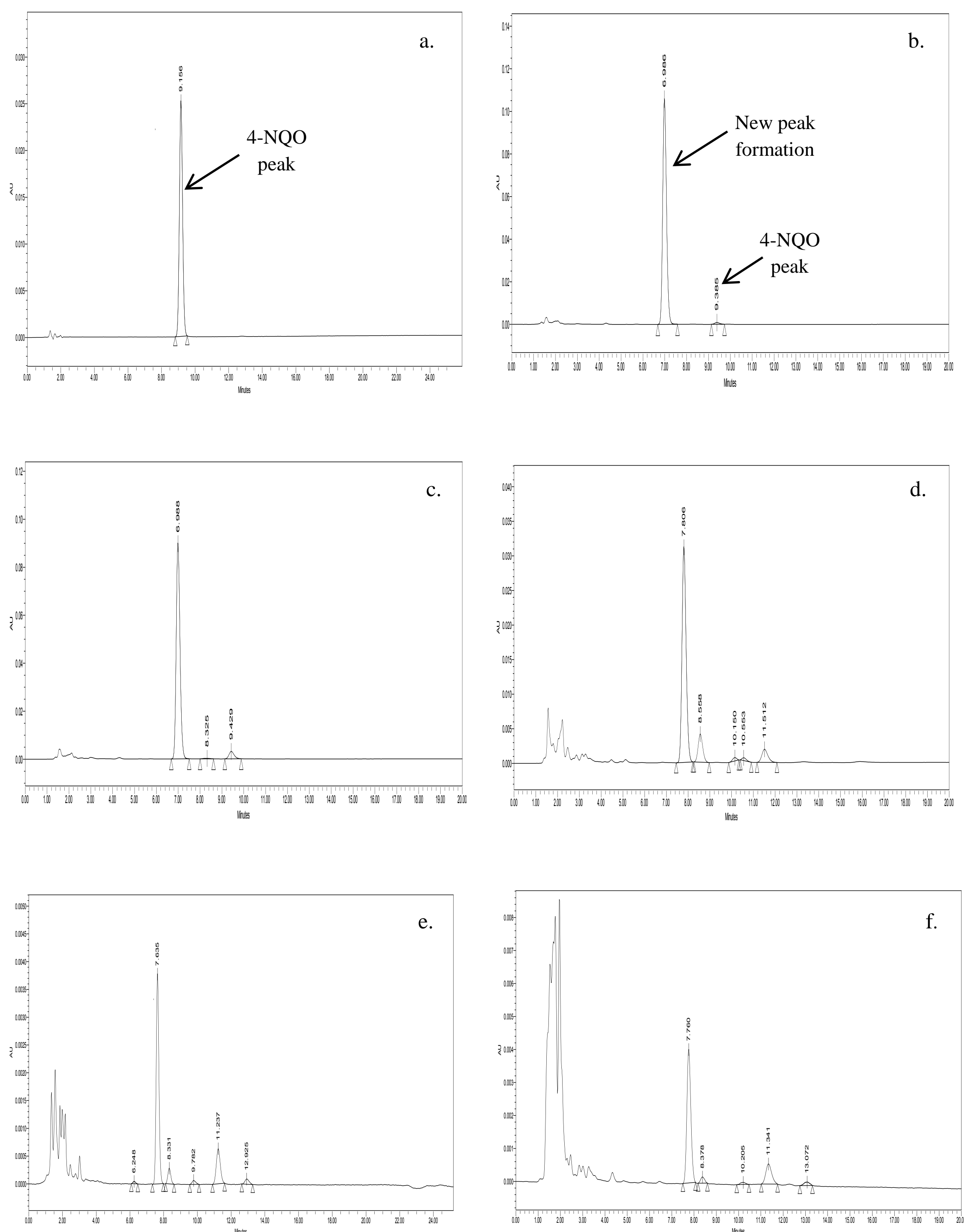

Fig. 5 HPLC chromatograms of supernatants of co-incubation assay mixture containing 4-NQO and cells of *L. rhamnosus* Fb analyzed at different time intervals [a] control 4-NQO ($RT_{9.156\ min}$), [b] 5 min ($RT_{6.986\ min}$, $RT_{9.835\ min}$), [c] 30 min ($RT_{6.988\ min}$, $RT_{8.325\ min}$ and $RT_{9.429\ min}$), [d] 180 min ($RT_{7.806\ min}$, $RT_{8.558\ min}$, $RT_{10.150\ min}$, and $RT_{11.512\ min}$), [e] 24 h ($RT_{6.248\ min}$, $RT_{7.635\ min}$, $RT_{8.331\ min}$, $RT_{9.782\ min}$, $RT_{11.237\ min}$ and $RT_{12.925\ min}$) and [f] 48 h ($RT_{7.760\ min}$, $RT_{8.378\ min}$, $RT_{10.205\ min}$, $RT_{11.341\ min}$ and $RT_{13.072\ min}$)

Discussion

Emerging role of probiotic bacteria in the protection and prevention of gastrointestinal infections and diseases prompted us to investigate their interaction with 4-NQO, a carcinogen often used in cancer-related studies (Yoshida *et al*., 2005). Here we provide experimental evidences of 4-NQO biotransformation by *Lactobacillus* strains, simultaneously leading to its detoxification reducing its DNA damaging activity. Antigenotoxicity of *Lactobacillus* strains with potential probiotic activities is advantageous as these are natural inhabitants of the human body mainly gastrointestinal tract.

Lactic acid bacteria isolated from dairy products such as yogurt, fermented milk and probiotic products have been reported to possess antigenotoxic and antimutagenic activities against 4-NQO (Lankaputhra and Shah, 1998; Pool-Zobel *et al*., 1996; Orrhage *et al*., 1994) reported antimutagenic activity of *L. acidophilus* strains and attributed it to their ability to produce organic acids especially butyric acid. Antigenotoxic and antimutagenic activities against 4-NQO of *Lactobacillus* strains isolated from fermented foods has been hypothetically associated with the biotransformation of 4-NQO (Cenci *et al*., 2002; Corsetti *et al*., 2008; Cenci *et al*., 2008). Walia *et al.* (2014) recently reported antigenotoxic and antimutagenic activities of *L. plantarum* and *L. fermentum* strains obtained from traditional fermented foods of North-Western Himalayas against 4-NQO.

In this context, our study provides conclusive evidence of 4-NQO biotransformation and detoxification by *L. rhamnosus* strains. This is evidenced in the (i) UV spectral modifications characterized by spectral shift towards shorter wavelength, and decreased absorbance at the λ_{max} of 4-NQO (in case of GG it increased), and (ii) appearance of new peak(s) with the simultaneous disappearance of the original peak in HPLC chromatogram. Disappearance of the new peaks formed upon prolonged incubation implies that the metabolites formed are degraded further purporting extensive degradation of 4-NQO. These modifications are tightly associated with the reduction in mutagenicity and genotoxicity evidenced from the two mechanistically distinct microbial assays SOS-Chromotest and Ames test. Biotransformation of 4-NQO occurs immediately upon addition of cells; the extent of 4-NQO biotransformation and simultaneous reduction in its genotoxicity and mutagenicity

increases with incubation time. Antigenotoxic activity of *Lactobacillus* strains was found to be higher against 4-NQO in comparison to its antimutagenic activity. Lactobacilli exhibited strain-specific 4-NQO biotransformation ability, the biotransformation products remain to be identified. The variations in the UV spectral modifications during 4-NQO biotransformation by the *Lactobacillus* strains provide indications that 4-NQO biotransformation is strain-specific though the strains belong to the same species. Heat-killed cells do not biotransform 4-NQO and exert antigenotoxicity, nonetheless, exhibited 40% of the antimutagenic activity shown by the untreated viable cells. Caldini *et al.* (2005) observed that heat-treated lactobacilli did not inactivate the genotoxin and suggested that the biotransformation of 4-NQO involves certain thermolabile components of cell wall and/or cell membrane. The biotransformation of 4-NQO is strictly related to the viability of the cells during co-incubation assay.

Biotransformation of 4-NQO by lactobacilli cells is influenced by cell density, pH and 4-NQO concentration. Biotransformation rate of 4-NQO is proportional to the number of the cells in the co-incubation mixture. Cell density-dependent antigenotoxic and antimutagenic activities of lactobacilli against 4-NQO provided additional evidence of cell-mediated biotransformation of 4-NQO. The disparity observed in the certain instances in the reduction in the genotoxicity and mutagenicity upon 4-NQO biotransformation perhaps results from the two mechanistically different test (SOS-Chromotest and Ames test *i.e*., *E. coli* PQ 37 and *S. typhimurium* TA 98) performed using two different organisms. The cell density-dependent antigenotoxicity is in accordance with the reports of other authors investigating the reduction of faecal water genotoxicity by the probiotic strains of *Bifidobacterium* spp. and *L. plantarum* (Burns and Rowland, 2000). It is difficult to determine if sufficient number of viable bacterial cells can be achieved in the human colon to have a significant effect on 4-NQO genotoxicity *in vivo*. However, our results showed that lactobacilli even at low cell density can exhibit DNA bio-protective activity in the gastrointestinal tract. Biotransformation of 4-NQO occurs at a lower rate in acidic pH (2-5), and higher in the neutral to alkaline pH range (6-10) indicates that 4-NQO would be rapidly biotransformed and detoxified in the intestine (pH~6-7), where the absorption of food is also maximum (Sreekumar and Hosono, 1998). *Lactobacillus* cells exhibit strain-specific concentration-dependent 4-NQO biotransformation rates and antigenotoxic

activity. Decreased antigenotoxic activity at higher concentration of 4-NQO may be related to reduced cell viability. 4-NQO biotransformation abilities of SGF-SIF-treated cells undoubtedly show that even in adverse conditions of gastrointestinal tract, lactobacilli can provide protection against potent carcinogens such as 4-NQO.

The cells subjected to various chemical treatments were used in co-incubation assay to identify the cell wall components involved in the binding and biotransformation of 4-NQO. HCl, $NaIO_4$, TCA treated cells demonstrated significantly lower antigenotoxic and antimutagenic activities. Decrease in antigenotoxic and antimutagenic activities by these treatments known to extract the cell surface carbohydrates (Heckles and Virji, 1988) indicates that cell surface carbohydrates play some role in the binding of 4-NQO. Divalent cations such as $MgCl_2$ and $CaCl_2$ reduced biotransformation of 4-NQO implicating involvement of teichoic acids in the biotransformation of 4-NQO. Inhibition of 4-NQO biotransformation with urea treated cells implicates the importance of hydrophobic interactions. Involvement of proteins is exemplified from the inhibition of 4-NQO biotransformation by NaOH and SDS treated cells. LiCl treatment known to extract the cell surface-associated proteins, did not exhibit any marked difference in biotransformation of 4-NQO, exclude involvement of surface layer proteins. Cell extract and cell debris did not exhibit antigenotoxic and antimutagenic activities ruling out involvement of intracellular component(s) and emphasize the importance of cell integrity in the biotransformation of 4-NQO. Inhibition of 4-NQO biotransformation and corresponding decrease in the antigenotoxicity and antimutagenicity with β-mercaptoethanol treated cells provides evidence for the involvement of thiol group containing metabolites e.g. GSH transferase - a detoxifying enzyme of Phase II metabolism, in the desmutagenesis of 4-NQO. GSH transferase activity of *Propionibacterium shermanii* has been implicated in the reduction of mutagenic activity of 4-NQO (Vorobjeva *et al*., 1996). However, more detailed studies are required to determine the role of thiol group and GSH transferase activity in the detoxification of 4-NQO by *L. rhamnosus* strains. Inhibitory influence of the treatment altering cell surface properties on the biotransfomation of 4-NQO implicates the importance of cell surface properties in the binding of 4-NQO for its subsequent biotransformation. Overall, experimental evidences indicate that the biotransformation is dependent on various factors such as the pH, cell density,

viability of the cells, 4-NQO concentration, and also the bacterial cell surface architecture.

The three lines of experimental evidences i) UV spectral modifications, ii) HPLC analysis and iii) microbial assays explicitly show that *Lactobacillus* strains biotransform 4-NQO simultaneously reducing its genotoxicity and mutagenicity. Thus, lactobacilli residing in the gastrointestinal tract play vital role against carcinogens by biotransforming them to inactive compound (s). Moreover, peptidoglycan and the cell wall carbohydrates and proteins form a part of the receptors facilitating the binding of 4-NQO. Potential probiotic candidates *L. rhamnosus* Fb and Vc exhibit the acid-bile and phenol tolerance, antimicrobial activity against harmful bacteria as well as fungi, also possess antigenotoxic and antimutagenic activities against 4-NQO. Novel strains *L. rhamnosus* Fb and *L. rhamnosus* Vc with respect to their ability to counteract the potential genotoxin provide important alternatives as food adjuncts and/or in probiotic formulations with additional health benefits. Further work is in progress to elucidate the mechanisms of 4-NQO biotransformation.