CHAPTER 6

Antigenotoxic and Antimutagenic Activities of *Lactobacillus rhamnosus* Vc against *N*-methyl-*N*'-nitro-*N*-nitrosoguanidine

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Introduction

Probiotic microbes are defined as 'the beneficial microbes which when ingested live in sufficient numbers improve the health of consumers' (Fuller, 1989). When the equilibrium of gut microflora is disturbed, the number of harmful organisms such as *Bacteroides*, and *Clostridia* and their toxic metabolites increases. Most of the probiotic strains such as *Lactobacillus* and *Bifidobacteria* are natural inhabitants of the human body and their fermentation products are claimed to exhibit antimutagenic and anticarcinogenic activities (Fernandes *et al*., 1987; Gilliland 1991). The enormous microbial diversity of the human gut microflora is reflected in a large and varied metabolic capacity, particularly in relation to xenobiotic biotransformation, and carcinogen synthesis and activation. The metabolic activities of the gut microflora can have wide-ranging implications, beneficial and detrimental effects on the health of the host (Hughes and Rowland, 2000). Probiotics and prebiotics gained a lot of attention as antimutagens for their carcinogen scavenging and elimination activity. In such condition, a food supplement/nutraceutical, principally rich in probiotics, prebiotics and synbiotics would serve as bio-protective agent in the removal of food-borne mutagens and carcinogens; thus preventing colorectal cancer (Raman *et al*., 2013). Probiotic bacteria have been shown to lower the enzymatic activities involved in carcinogen formation such as azoreductase, nitroreductase, β-glucuronidase, and metabolism in comparison to other major anaerobes in the gut such as *Bacteroides*, and *Clostridia* (Saito *et al*., 1992). This suggests that increasing the proportion of lactobacilli in the gut could beneficially modify the levels of xenobiotic-metabolizing enzymes. Many *N*-nitroso compounds (NOC) formed during metabolism are potent DNA alkylating agents and exert carcinogenic-mutagenic effects. NOCs have been related to an increased risk of gastric cancer (Xu and Reed, 1993). Nitrosoable mutagen precursors are found in beer, pepper, tobacco products, soybean, cured meat, fermentation products, vegetables and various types of cooked foods (Wakabayashi *et al*., 1989). Preformed NOCs are found in cosmetics, pharmaceutical products and

occupational sources (Hughes and Rowland, 2000). In addition to these exogenous sources, there is also a major possibility of endogenous formation of *N*-nitroso compounds by the reaction of nitrite with secondary amines and amides (Ohshima and Bartsch, 1981). *N*-nitrosation may be acid-catalyzed or bacterial-catalyzed at a neutral pH. Therefore, NOC formation may occur at a number of sites in the body (Massey *et al*., 1988). The large intestine is rich in nitrogenous residues and nitrosating agents from protein metabolism and dissimilatory nitrate metabolism respectively providing site for *N*-nitrosation reactions (Macfarlane and Cummings, 1991). Nitrogenous residues entering the colon increase with increasing protein intake (Silvester and Cummings, 1995), and are available for *N*-nitrosation by colonic bacteria via nitrite and nitrate reductase (Calmels *et al*., 1985).

The studies have been reported describing the antimutagenic activity of *Lactobacillus* strains against MNNG (Lankaputhra and Shah 1998; Caldini *et al*., 2005; Ambalam *et al*., 2011). However, there is very scarce information regarding antigenotoxic and antimutagenic activities of *L. rhamnosus* against MNNG. Here we report the *in vitro* biotransformation of MNNG by human origin *L. rhamnosus* Vc.

Materials and Methods

Chemicals

N-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) was obtained from Sigma (USA). Stock solution of MNNG (1 mg/ml) was prepared in sterile distilled water, and stored at 4°C. Working solution was prepared just before testing. *o*-nitrophenyl-β-D-galactopyranoside (ONPG), *p*-nitrophenyl phosphate (PNPP) (Sigma, USA).

Bacterial strains

L. rhamnosus Vc strain was isolated from vaginal mucosa of healthy female. The strain was selected because of its probiotic properties such as, acid-bile, NaCl, phenol tolerance, autoaggregation and coaggregation abilities, and antagonistic activity against intestinal and vaginal pathogens (Pithva *et al*., 2014). The stock culture was maintained in 10% (w/v) skim milk at 4°C. *Salmonella typhimurium* (*his*$^-$) TA-98 used for mutagenicity was grown in Nutrient broth II (Himedia, India) for 18 h at 37°C and obtained as a kind gift from (Dr. Ramadasan Kuttan, Amala Cancer Research Center, Kerala, India). *Escherichia coli* PQ37 (*sfiA::lacZ*) for genotoxicity

assay was grown in Luria broth (Himedia, India) for 12-15 h at 37°C and procured from Institute Pasteur (Paris, France).

In vitro inhibition of genotoxic and mutagenic activities of MNNG by *L. rhamnosus* Vc

24 h old cells of *L. rhamnosus* Vc grown in MRS broth at 37°C were pelleted by centrifugation (5000 rpm, 15 min at 4°C), washed twice with PBS and resuspended in phosphate buffer (0.1 M, pH 7.0). The co-incubation assay mixture, in a total volume of 1.0 ml contained 800 µl phosphate buffer (0.1 M, pH 7.0), 100 µl MNNG (68 µM), and cell suspension (OD_{600} = 1.0 *ca.* 10^9 cfu/ml), was incubated on shaker (90-100 rpm, 37°C, 180 min), centrifuged (6000 rpm, 10 min, 4°C) and the supernatant was filter sterilized using 0.22 µm pore size (Millipore) and scanned (200-400 nm) using UV-Visible spectrophotometer (UV1601, Shimadzu, Japan). 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 MNNG) as 100%.

Influence of pH, incubation time, cell density, and MNNG concentration on antigenotoxic and antimutagenic activities of L. rhamnosus Vc against MNNG

Co-incubation assay was carried out as described above. The assay mixture was prepared in 100 mM glycine-HCl (pH 2.0, 3.0), acetate (pH 4.0, 5.0), phosphate (pH 6.0, 7.0), tris (pH 8.0, 9.0) and glycine-NaOH (pH 10) buffers. Co-incubation assay was performed varying (i) incubation time (30-180 min), (ii) cell density (OD_{600} = 0.1, 0.5, 1.0 and 2.0), and (iii) MNNG concentration (68-340 µM). The supernatants were analyzed as described above.

Genotoxicity assay: SOS-Chromotest

The assay is based on the activation of SOS-response in *E. coli* PQ37 strain Quillardet *et al*. (1985) that carries *sfiA::lacZ* gene fusion and is *lac*$^-$. β-galactosidase activity is therefore strictly dependent on *sfiA* expression. *E. coli* PQ 37 culture (0.1 ml) grown overnight in Luria broth plus ampicillin (20 µg/ml) was transferred to 5.0 ml of Luria broth containing ampicillin and incubated on shaker for 2 h (100 rpm, 37°C). The OD_{600} of this culture should be between 0.3-0.4. 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 25 µl supernatant from the co-incubation assay or MNNG (68 µM), was incubated for 2 h (100 rpm, 37°C). After incubation, 100 µl of the above reaction mixture was taken into two series of tubes and simultaneously analyzed for β-galactosidase and alkaline phosphatase activities colorimetrically at 420 nm using ONPG and PNPP respectively. Positive and negative controls were prepared in phosphate buffer with or without MNNG, respectively. SOS induction factor (IF_{SOS}) = [(β-galactosidase activity/alkaline phosphatase-test)/ [(β-galactosidase activity/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

The mutagenicity was estimated by measuring the extent of reverse mutation of *Salmonella typhimurium* (*his*$^-$*)* TA 98 auxotroph strain to prototroph as described by (Ames *et al*. 1975; Mortelmans and Zeiger, 2000). Briefly, 100 µl of an overnight grown culture of the 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 20 µl MNNG (positive control), and/or supernatant from co-incubation assay mixture as described above. The mixture was pre-incubated at 37°C for 20 min, then mixed with 2.0 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. Antimutagenic activity (%) was calculated using the formula (C_R-T_R/C_R) x 100, where C_R is revertant colonies in control, T_R is revertant colonies in treated.

Pretreatment of lactobacilli

Cells were pelleted by centrifugation (5000 rpm, 15 min, 4°C) from 24 h *L. rhamnosus* Vc 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

(1998). 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.

Biotransformation of MNNG by 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 (10000 rpm, 25 min, 4°C). Supernatant was filtered through 0.22 µm filter (Millipore) and used as cell extract for co-incubation assay instead of cell suspension.

Biotransformation of MNNG 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 co-incubation assay with MNNG.

Statistical Analysis

The results are represented as mean and standard deviations. Difference between the mean values was 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 incubation time and cell densities was calculated.

Results

In vitro inhibition of MNNG genotoxic and mutagenic activities by *L. rhamnosus* Vc

The genotoxic and mutagenic activities of MNNG was reduced to 69 and 61% after 3 h of co-incubation with non-growing viable cells of *L. rhamnosus* Vc. The *in vitro* evidence of *L. rhamnosus* Vc inhibiting genotoxic and mutagenic activities of MNNG

were in agreement with two distinct microbial assays SOS-Chromotest and Ames test. Inhibition of MNNG activities was accompanied with UV spectral modifications of the parent compound, characterized by shift in absorbance maxima of MNNG λ_{268} to shorter wavelength λ_{264}, and increase in the absorbance at λ_{max} (Fig. 1). Heat treatment of *L. rhamnosus* Vc cells before exposure to MNNG completely inhibited the cells antigenotoxic and antimutagenic activities, and also failed to induce modifications in the UV spectrum of MNNG (Fig. 1). *L. rhamnosus* Vc cells upon co-incubation with 68-340 µM MNNG for 3 h exhibited reduction in viability, ranging from 16-71% (Fig. 2).

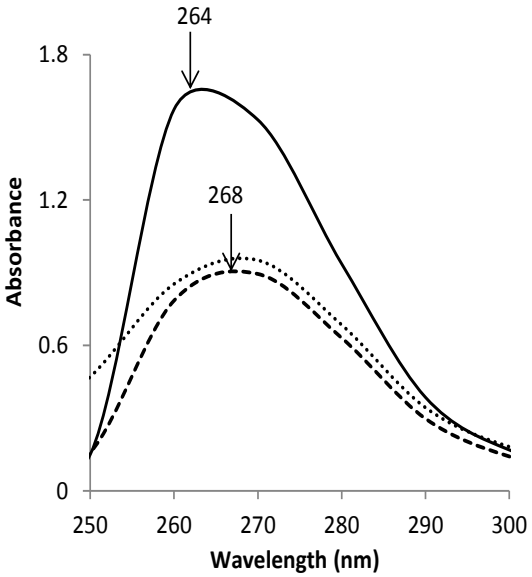

Fig. 1 UV spectra of MNNG (68 µM) after co-incubation of 3 h with *L. rhamnosus* Vc, dashed line shows control MNNG (--) without *L. rhamnosus* Vc cells, *L. rhamnosus* Vc (-) and (**…**) heat-killed cells

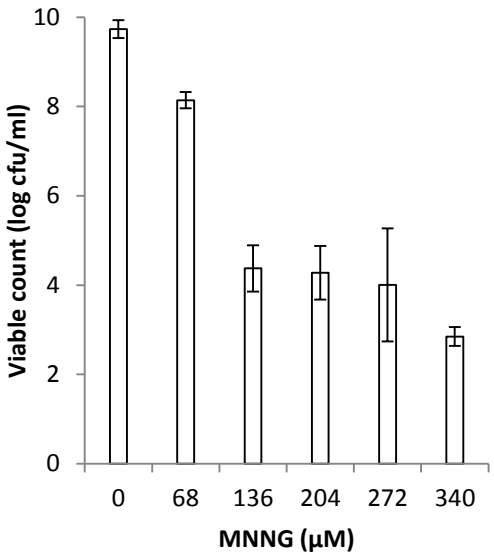

Fig. 2 Viable count (log cfu/ml) of *L. rhamnosus* Vc after 3 h of co-incubation with 0-340 µM MNNG; viability was determined by viable count method using MRS medium. Error bars indicate standard deviation

Influence of pH, incubation time, cell density, and MNNG concentration on antigenotoxic and antimutagenic activities of L. rhamnosus Vc against MNNG

pH of the co-incubation assay mixture influenced the extent of spectral modifications and the genotoxicity inhibition of the added MNNG. Antigenotoxic activity was higher at pH 7 than at other pHs tested (Fig. 3) and so were the extent of spectral modifications.

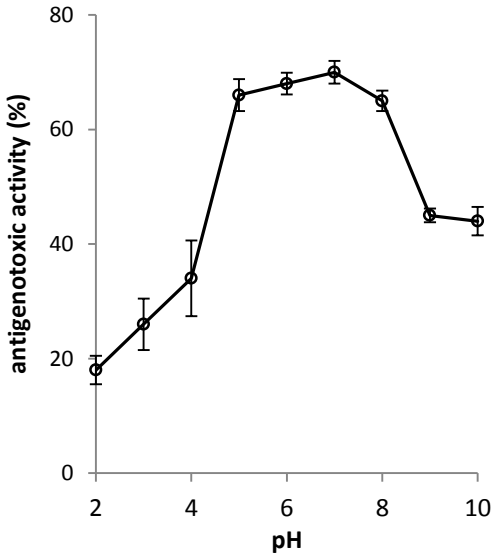

Fig. 3 Antigenotoxic activity (%) of *L. rhamnosus* Vc against MNNG at pH 2-10 evaluated by SOS-Chromotest, error bars indicate standard deviation

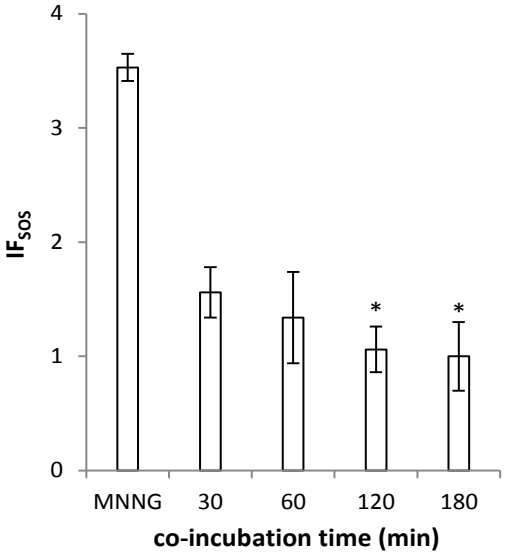

Fig. 4 SOS-induction factor (IF_{SOS}) of MNNG after co-incubation (30-180 min) with *L. rhamnosus* Vc cells evaluated by SOS-Chromotest, error bars indicate standard deviation *not significantly different (*P*=0.055)

Correlation was observed between (r=0.75) antigenotoxic activity and incubation time. Genotoxicity of MNNG gradually decreased as the incubation time was increased from 30 to 180 min. IF_{sos} decreased from 3.53 to 1.56 at 30 min to 1.00 at 180 min of co-incubation (Fig. 4). Genotoxic inhibition, upon co-incubation of *L. rhamnosus* Vc at various cell densities (OD_{600} = 0.1, 0.5, 1.0 and 2.0) with 68 µM MNNG, increased proportionally with increasing cell densities (Fig. 5) Antigenotoxic activity and cell density of *L. rhamnosus* Vc were highly correlated (r=0.82). IF_{sos} of MNNG 3.53 significantly (*P*=0.028) decreased to 1.56, 1.26, 1.09 with the cell densities of 0.1, 0.5, and 1.0 respectively. Further increase in cell density from 1.0 to2.0 did not influence IF_{sos} (*P*=0.290). Antimutagenic activity of *L. rhamnosus* Vc was similar even with various cell densities, incubation times and pHs (results not shown). Antigenotoxic activity evaluated at 68-340 µM MNNG concentration, with the same cell density 10^9 cfu/ml for 180 min demonstrated negative correlation. As the concentration increased from 68-340 µM, there was gradual decrease in the antigenotoxic activity from 60 to 17% (Fig. 6a).

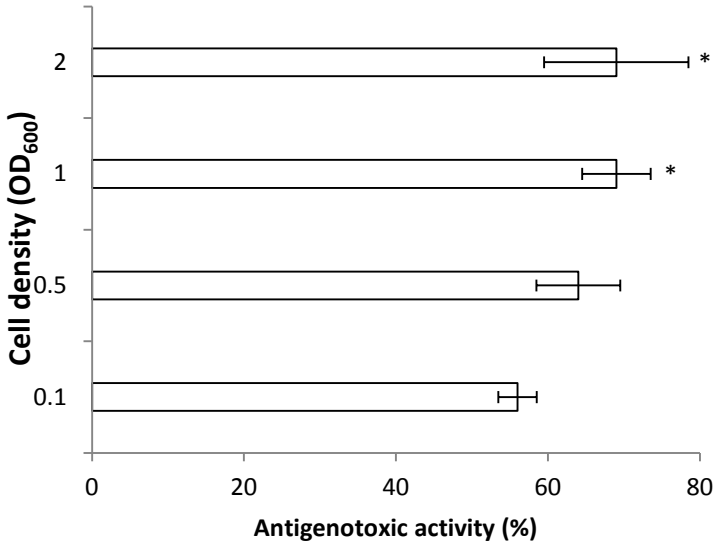

Fig. 5 Antigenotoxic activity (%) of *L. rhamnosus* Vc at cell densities (OD_{600} = 0.1, 0.5, 1.0, 2.0) against MNNG determined by SOS-Chromotest. error bars indicate standard deviation (n=3), *not significantly different (*P*=0.29)

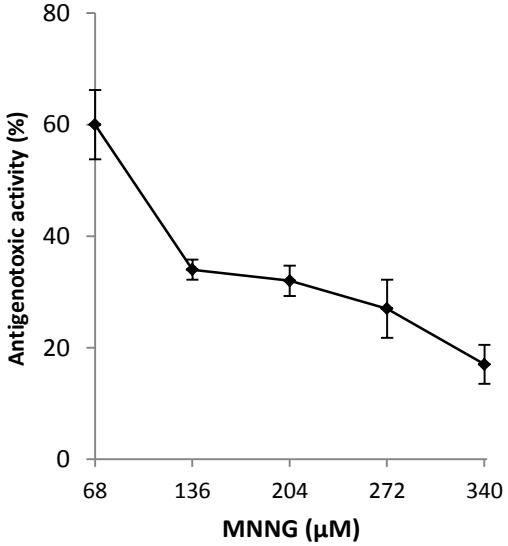

Fig. 6a Antigenotoxic activity (%) of *L. rhamnosus* Vc against MNNG (68-340 µM) determined after 60 min of co-incubation, error bars indicate standard deviation

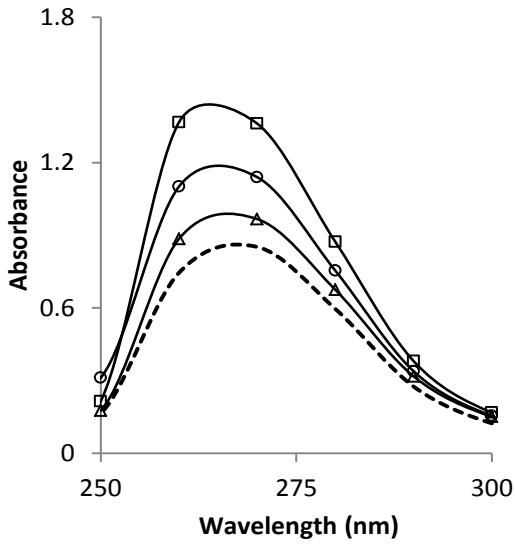

Fig. 6b UV spectra of (---) MNNG after co-incubation with (□) untreated cells, (○) SGF-treated and (Δ) SGF-SIF-treated cell

Simulated gastrointestinal fluid treated cells did not significantly alter MNNG biotransformation abilities (Fig. 6b), although occur at lower rate in comparison to untreated cells.

Pre-treatments influence the carbohydrate component of the cells caused inhibition of spectral shift as evidenced from the HCl, $MgCl_2$, $CaCl_2$, $NalO_4$ and TCA treated cells. Protein-denaturing treatment such as NaOH, SDS, and LiCl inhibited the spectral shift in comparison to untreated cells. Moreover, urea is an anti-hydrophobic agent caused the inhibition of spectral shift (Fig. 7). Surface layer protein extract evidently exhibited significant UV spectral modification in MNNG spectrum (Fig. 8)

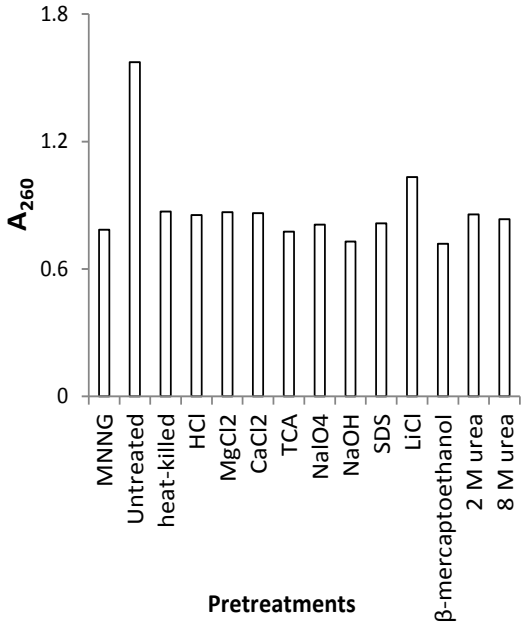

Fig. 7 Changes in A_{260} of MNNG upon co-incubation with pretreated cells

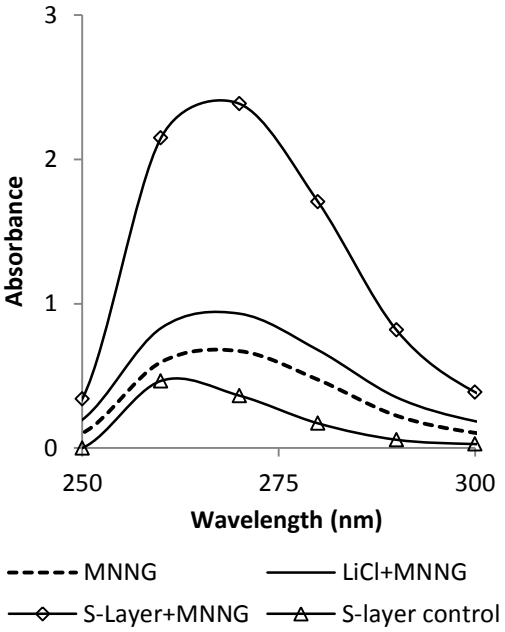

Fig. 8 UV spectra of MNNG upon co-incubation with surface-associated protein extract of *L. rhamnosus* Vc

Discussion

The emerging relationship between the gut microbiota and colon cancer (Compare and Nardone, 2011) provides a new opportunity for colon cancer protection by means of feeding live probiotic bacterial cells. Evidence from epidemiological and experimental studies implies that diet and intestinal micro flora are important in the etiology of colon cancer. Probiotic lactic acid bacteria can change the colonic microbiota that might prevent diseases by means of maintaining the homeostatis between the beneficial and harmful bacteria. MNNG, a direct-acting alkylating agent that methylates the DNA, is a potent carcinogen causing colon cancer (IARC monographs, 1974). Previous studies describing *in vitro* antimutagenic activity of

lactic acid bacteria have largely used *L. acidophilus* strains. Hosoda *et al*. (1992) evaluated antimutagenic activity of milk cultured with different *Lactobacillus* strains against MNNG employing Ames test *viz*. *L. helveticus* (15.7-52.8%), *L. delbrueckcii* (14.9-57.8%), *L. acidophilus* (27.6-77.0%), *L. casei* (57-61%), *L. rhamnosus* (92.7-55.8%), *L. salivarius* (47%) and *L. plantarum* (26.9-27.6%). Nadathur *et al*. (1994) reported 59-95% reduction in mutagenicity of MNNG by the extracts of milk fermented with *L. acidophilus*. Lankaputhra and Shah (1998) reported 10-50% reduction in the mutagenic activity of MNNG by the *L. acidophilus* strains. Caldini *et al*. (2005) reported 95% antigenotoxic activity of *L. acidophilus* A9 against MNNG using SOS-Chromotest. Ambalam *et al*. (2011) evaluated the binding and antimutagenic activity of *L. rhamnosus* 231 against MNNG and showed 77% reduction in mutagenicity by Ames test.

Our present findings provide conclusive evidences of the *in vitro* and *in vivo* protective effects of probiotic *Lactobacillus rhamnosus* Vc (Pithva, 2008) against severity of MNNG-induced colon inflammation. *L. rhamnosus* Vc cells upon co-incubation biotransformed MNNG evidenced from the UV spectral modifications and simultaneous reduction in genotoxicity and mutagenicity evaluated by two most widely used microbial assays SOS-Chromotest and Ames test. Live bacterial cells caused extracellular modification in the structure of MNNG converting it to non-toxic form(s) reducing the DNA-damaging activity in comparison to the parent compound. Moreover, viability of bacterial cells is an important prerequisite for the biotransformation of MNNG as heat-killed cells do not induce UV spectral modification and do not exhibit antigenotoxic and antimutagenic activities.

Several lines of evidences, apart from the inactivity of heat-killed cells, exert the importance of the cells viability in MNNG biotransformation and detoxification, i.e., (i) extent of biotransformation and the decrease in genotoxicity at various pHs, incubation time and cell densities; the extent of MNNG biotransformation proportionally decreased its genotoxicity. Higher antigenotoxic activity of *L. rhamnosus* Vc in the pH range 5.0-8.0 implies that MNNG would be optimally and rapidly biotransformed and detoxified in the intestine where the pH is ~6-7 (Sreekumar and Hosono, 1998). Such strains can provide protection against endogenously formed MNNG, a major cause of colon carcinoma. The ability of the *L.*

rhamnosus Vc cells to biotransform MNNG within 30 min is further significant as it will biotransform MNNG immediately upon its formation permitting little for its absorption. In this context, the resident time of bacterial cells in the intestinal tract, which is approximately 3 h the time required for food passage time (Vitali *et al*., 2012) before the washout of cells begin will ensure biotransformation of MNNG and excretion of biotransformation product(s) via feces in the non-toxic form. MNNG biotransformation is indeed a biochemical interaction mediated by the live bacterial cells, exemplified in the correlationship observed between UV spectral modifications and genotoxic inhibition, with cell viability, cell densities, incubation time and pH. Population of probiotic bacteria in the gastrointestinal tract varies from person to person due to food habits (Lozupone *et al*., 2012) under such conditions probiotic strain even at a lower cell density possess the ability to counteract carcinogens such as MNNG, and would act beneficially as a DNA-bioprotective agent. Such protection further enhanced by supplementing the probiotic *L. rhamnosus* Vc in day to day food as supplement. As we observed in our previous studies that feeding of *L. rhamnosus* Vc to the chicks provided the protection against MNNG-induced inflammation and evidenced from glutathione *S*-transferase activity. Moreover, retention of MNNG biotransformation abilities with simulated gastrointestinal fluid treated cells signifies that bacteria residing in the GIT provides the protection against endogenously formed mutagens such as MNNG. Pretreatment of *L. rhamnosus* Vc cells with various chemicals revealed that cell wall components such as carbohydrates, proteins and teichoic acids and hydrophobic interactions are involved in MNNG biotransformation. Surface-associated proteins are evidently involved in MNNG biotransformation as the LiCl-treated cells inhibited MNNG biotransformation. Inhibition of MNNG biotransformation 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 MNNG. However, more detailed studies are required to determine the role of thiol group and GSH transferase activity in the detoxification of MNNG by *L. rhamnosus* Vc.

From the above findings, it can be concluded that probiotic *L. rhamnosus* Vc is a potential DNA-protective agent against the MNNG-induced colon inflammation mainly because of its interaction with MNNG resulting in its biotransformation and detoxification. Biotransformation leads to the conversion in to less toxic and inactive

compound(s). Moreover, cell wall carbohydrates, teichoic acid and proteins facilitating the MNNG biotransformation. Detoxification has been evidenced from the microbial assays SOS-Chromotest, Ames test. Direct and indirect evidences indicate probiotics are promising dietary supplement that confer prophylactic therapeutic benefits apart from the beneficial activities such as protection against pathogens. Further studies addressing the mechanisms of antimutagenesis of *L. rhamnosus* Vc against endogenous formation of carcinogenic compounds such as MNNG are in progress.