8 Binding and Biotransformation of Heterocyclic Aromatic Amine 2-amino-3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (MeIQx) by *Lactobacillus* strains

Introduction

A number of dietary components have been implicated as possible causative factors in the etiology of human cancer based human epidemiology and high-dose animal studies (Wakabayashi *et al*., 1992; Thorogood *et al*., 1994). Among 20 heterocyclic aromatic amines 2-amino-3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline is one such dietary component that has been isolated from cooked protein products, particularly red meat, fish and poultry. MeIQx levels in these products have been reported to be in the range of 1 to 30 ppb, with daily consumption as high as several µg heterocyclic amine/person/day (Felton and Knize 1990; Layton *et al*., 1995). Heterocyclic aromatic amines (HCA) are compounds with high mutagenic potential, formed when meat is cooked at high temperatures 150-300°C. These compounds contribute to the development of colon and gastric cancer. Western diet provides a lot of HCA to direct acting mutagens (Nowak and Libudzisz, 2009). Colon cancer was enhanced in animals fed with well-cooked meat containing high levels of HCA. Several studies have claimed relationship between higher consumption of well-done meat and the risk of colon, breast, lung and gastric cancer (Terry *et al*., 2003; Oba *et al*., 2006; Wu *et al*., 2006; O'Keefe *et al*., 2007; Shin *et al*., 2007). Western diet high in protein content has demonstrated carcinogenic properties and affects the substrates available to the gut microbiota. Intestinal microbiota can activate HCA to their active derivatives. The major anaerobic metabolites of MeIQx after incubation with mixed human faecal microbiota was 2-amino-3,6-dihydro-3,8-dimethylimidazo[4,5-*f*]quinoxalin-7-one, which is direct acting mutagens (Humbolt *et al*., 2005; Van Tassell *et al*., 1990). The human gastrointestinal tract possesses a complex ecosystem, the components of which are generically complex and metabolically diverse with bacterial number reaching 10^{11}/g intestinal contents in the large intestine (Cummings, 1997). The principal role of the gut microflora is to save energy from non-digested dietary substrates and endogenous mucus during fermentation. The nature and extent of this metabolism

depends upon the characteristics of the bacterial flora, colonic transit time and the availability of nutrients especially carbohydrates and proteins. Products of carbohydrate fermentation are thought to benefit the host in contrast to the potentially toxic products of protein fermentation. The metabolic activities of the gut microflora have also been associated with cancer development following the formation of toxic products by bacterial enzyme activities (Hughes and Rowland, 2000). The indigenous bacteria have been classified as either potentially harmful or health promoting strains with beneficial properties, which are potential sources of probiotics, most frequently belong to the genera *Bifidobacterium* and *Lactobacillus* (Isolauri, 2004; Limdi *et al*., 2006). On the other hand intestinal microbiota are also able to produce many toxic, potentially carcinogenic substances, which can contribute to colon cancer by activating genotoxic and carcinogenic substances, and converting procarcinogens to electrophiles, which can easily react with DNA (Burns and Rowland, 2000). Potential mechanisms underlying anticarcinogenic action of lactic acid bacteria living in the colon may include the inhibition of colonic enzyme activity, control of growth of other 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 (Blaut *et al*., 2006; 2007; O'Hara and Shanahan, 2007). Present study provides the potential of human origin strains to interact with potent heterocyclic aromatic amine MeIQx that resulted in the biotransformation.

Materials and Methods

Bacterial strains and culture condition

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 (Pithva *et al*., 2014) Reference strains 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% skim milk at 4°C.

Carcinogen

2-amino-3,8-dimethyl-3*H*-imidazo[4,5-*f*]quinoxaline (CAS No. 77500-04-0) was purchased from Toronto Research Chemicals (Canada). MeIQx was dissolved in DMSO at the final concentration of 10 mg/ml, and stored at 4°C.

Binding assay in phosphate buffer

The cells were obtained from the 24 h old cultures of lactobacilli grown in MRS medium by centrifugation (5000 rpm, 15 min, 4°C), washed twice with PBS and resuspended in phosphate buffer (0.1 M, pH 7). Reaction mixture comprised of 800 µl phosphate buffer, cell suspension (OD_{600} = 1, 10^9 cfu/ml) and 100 µl of MeIQx at a final concentration of 30 µg/ml. The assay mixture was incubated on shaker (90 rpm, 37°C) for 30 min, centrifuged (6000 rpm, 10 min at 4°C), and the supernatant collected was scanned (200-400 nm) using UV-Visible spectrophotometer (UV1601, Shimadzu, Japan). The positive control consisted of phosphate buffer with 30 µg/ml of MeIQx and negative control contained cell suspension of *Lactobacillus* strains without MeIQx. In order to check the influence of various physical factors on MeIQx binding and biotransformation following parameters were studied. The reaction assay mixture was prepared in 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. Assay was performed varying (i) incubation time (30-180 min), (ii) cell density (OD_{600} = 0.1, 0.5, 1 and 2), and (iii) heat-killed cells (100°C for 15 and 30 min) The supernatant was analysed for residual MeIQx by scanning UV-Visible spectrophotometer.

Simulated gastrointestinal 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 MeIQx.

Influence of pretreated lactobacilli cells on MeIQx 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 treatments 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 by Sreekumar and Hosono (1998b). 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 µ filter (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 with MeIQx

Surface-associated protein extract and intracellular cell extracts were used for co-incubation assay with MeIQx instead of cell suspension 900 µl extract co-incubated with MeIQx.

High performance liquid chromatography

MeIQx was assayed by HPLC (Shimadzu) equipped with a photo diode array detector. The C_{18} column was used for the MeIQx assay. The mobile phase consisted of acetonitrile:water (50:50); at the flow rate of 0.5 ml/min and absorbance was monitored at 254, 274 and 342 nm.

Results

Adsorption of MeIQx by Lactobacillus strains

Resting cell preparation of lactobacilli upon co-incubation with 30 µg/ml of MeIQx in phosphate buffer caused 58-63% adsorption of MeIQx in initial 30 min of incubation, which further leading to the biotransformation of MeIQx that was evidenced from the UV spectral modification λ_{274} shifted to λ_{269} with increase in absorbance (Fig. 1a).

The UV spectral modification was more prominent at 180 min of incubation (Fig. 1b). Moreover, amount of MeIQx adsorbed remained constant with increased incubation time from 30 to 180 min (Fig. 1c). MeIQx adsorption remain steady beyond cell density (OD_{600}>0.1) (Fig. 2). pH significantly influenced the MeIQx biotransformation, increased A_{270} was higher in the pH range 7-10 (Fig. 3).

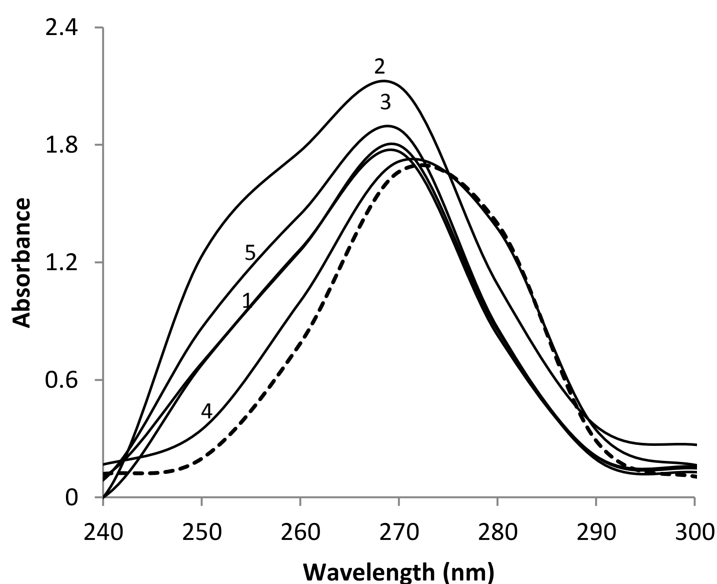


Fig. 1a UV spectral modification of MeIQx after co-incubation of 3 h with *Lactobacillus* strains control MeIQx - without bacterial cells (dashed line), 1. *L. rhamnosus* Fb, 2. *L. rhamnosus* GG, 3. *L. rhamnosus* 231, 4. *L. rhamnosus* Vc, and 5. *L. casei* Actimel

Absorbance

a b c d e

MeIQx
30 min
60 min
120 min
180 min

Wavelength (nm)

Fig. 1b UV spectra of MeIQx co-incubated for different time interval (30-180 min) with a) *L. rhamnosus* Fb, b) *L. rhamnosus* GG, c) *L. rhamnosus* 231, d) *L. rhamnosus* Vc and e) *L. casei* Actimel

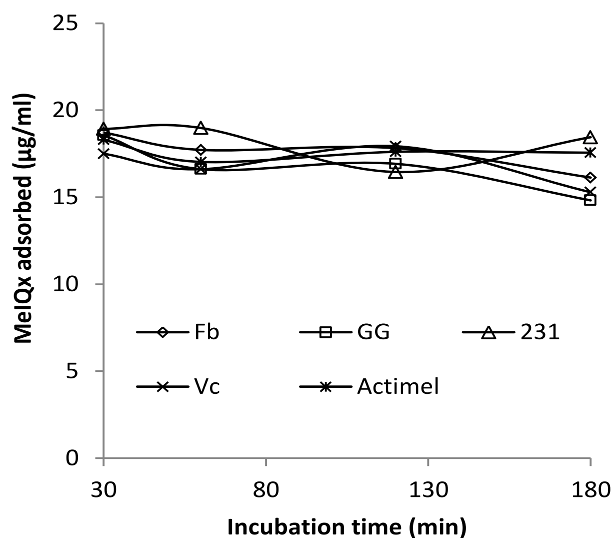


Fig. 1c MeIQx adsorbed (µg/ml) by *Lactobacillus* strains incubated for 30-180 min

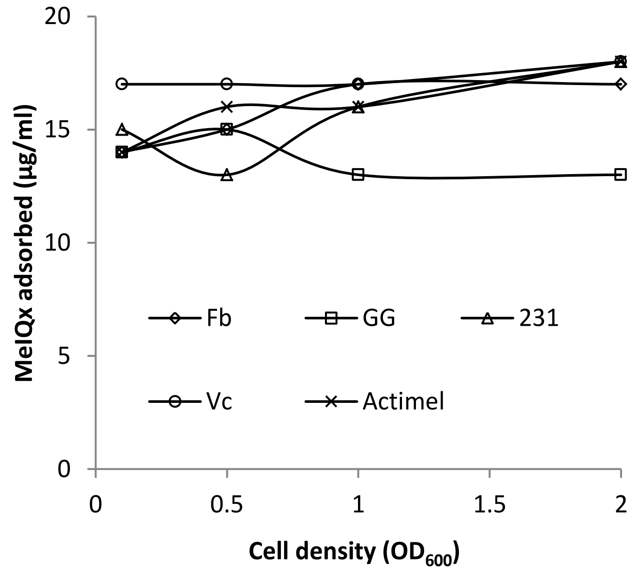


Fig. 2 Influence of cell density (OD_{600}) on MeIQx adsorption (μg/ml) by *Lactobacillus* strains

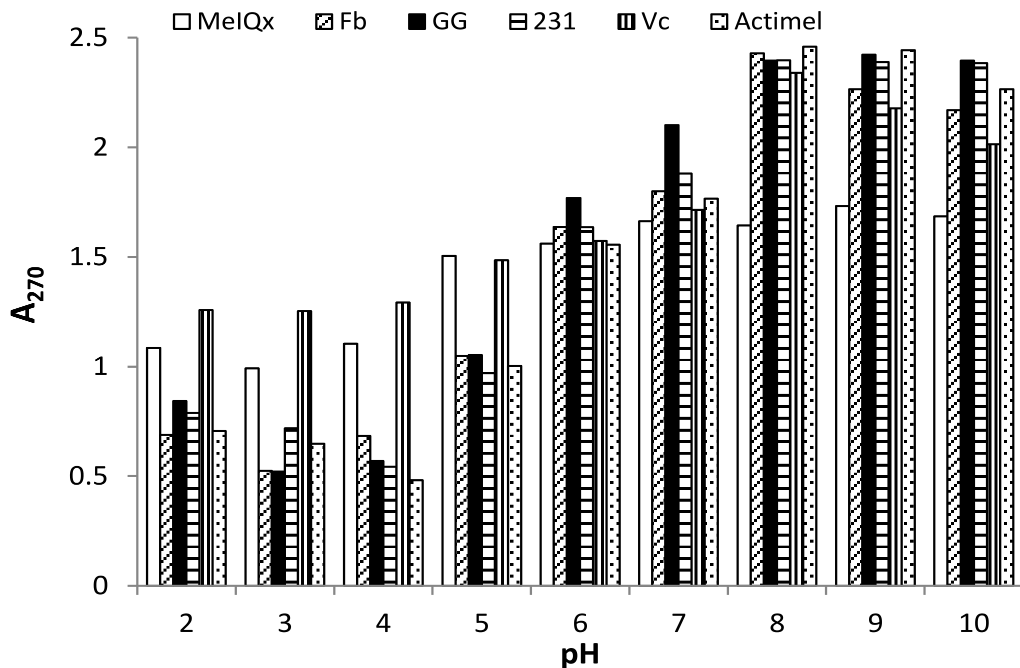


Fig. 3 Changes in A_{270} of MeIQx upon co-incubation with *Lactobacillus* strains at different pH (2-10)

Simulated gastrointestinal fluid treated cells did not significantly alter MeIQx adsorption abilities (Fig. 4). Another inference is A_{270} spectral shift decreased with SGF and SGF-SIF-treated cells. Heat-killed cells did not influence the MeIQx spectrum. The pre-treatments influence the carbohydrate component of the cells cause 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 treatment is an anti-hydrophobic agent and breaks hydrogen bond caused the inhibition of spectral shift (Fig. 5a). Surface layer protein extract evidently exhibited significant UV spectral modification in MeIQx spectrum (Fig. 5b).

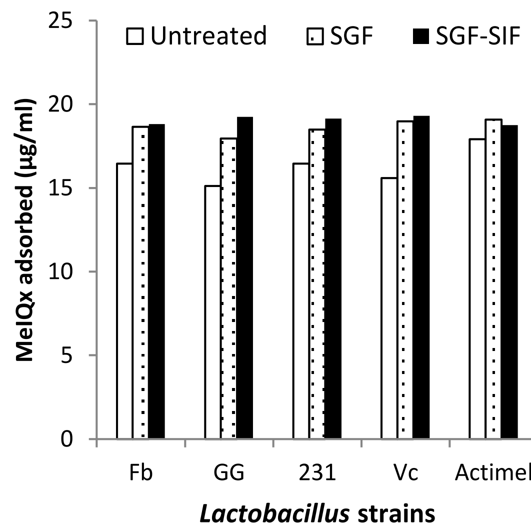


Fig. 4 MeIQx adsorption by untreated cells, SGF treated and SGF-SIF-treated cells of *Lactobacillus* strains

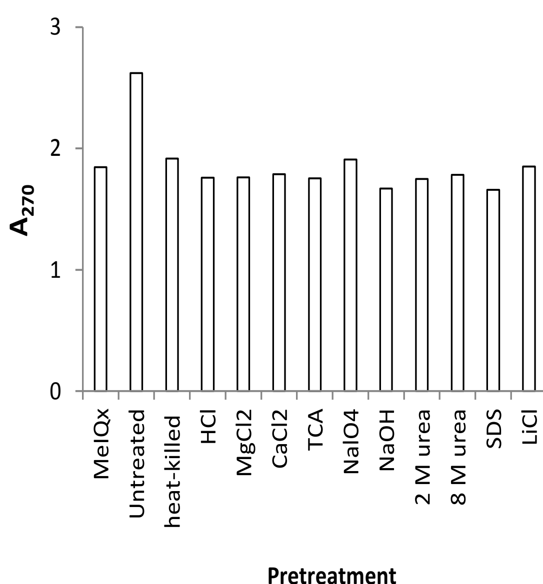


Fig. 5a Changes in A_{270} of MeIQx upon co-incubation with pretreated cells

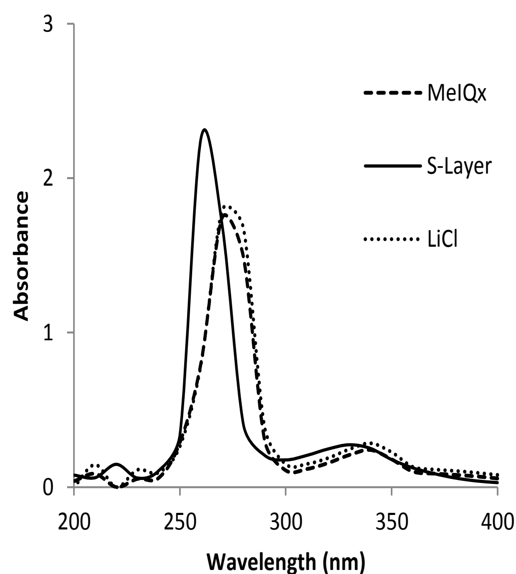
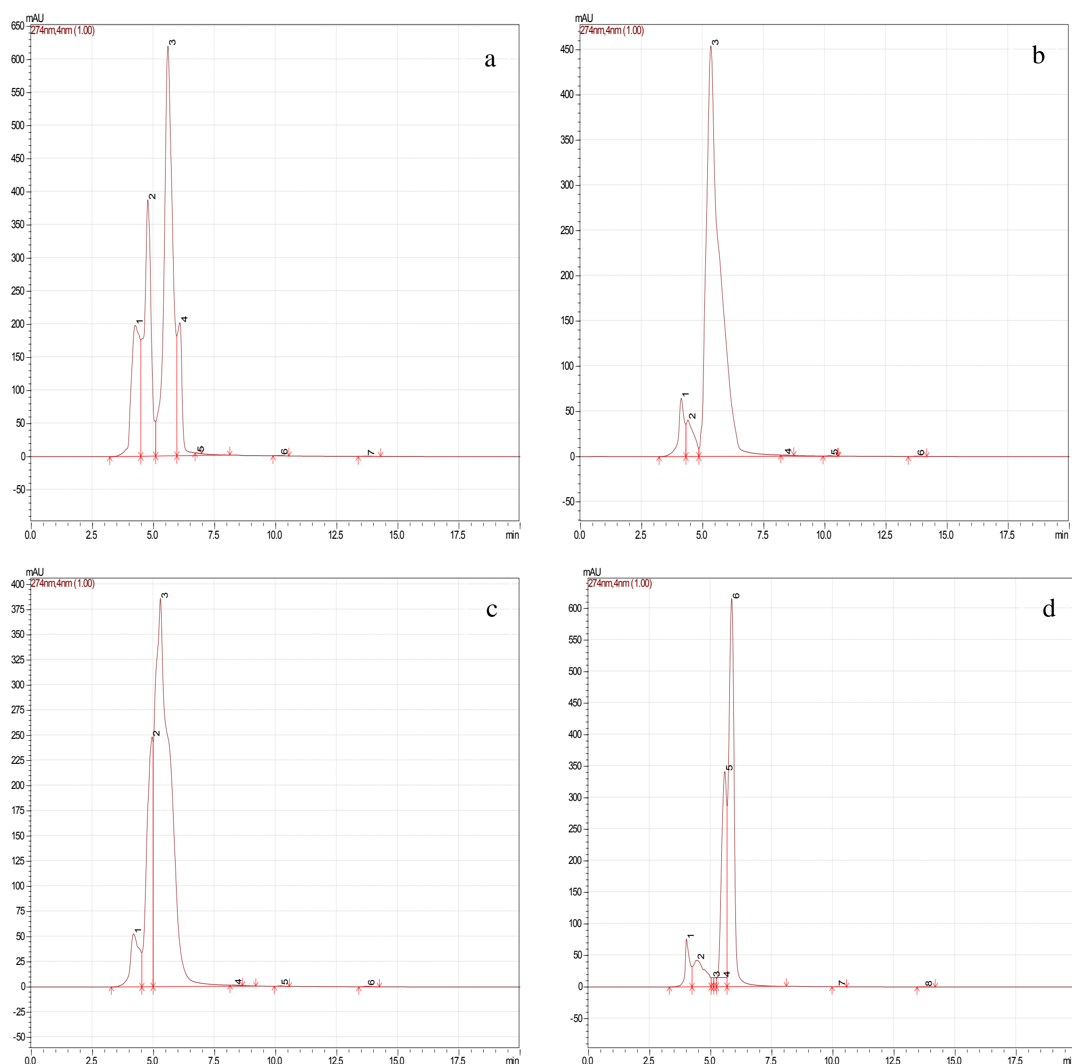


Fig. 5b MeIQx biotransformation by surface-associated protein extract of *L. rhamnosus* Fb

High performance liquid chromatography

HPLC analysis provided additional evidence of MeIQx biotransformation. The major peak of the parent compound ($RT_{5.581}$) along with the minor peaks ($RT_{4.251}$, $RT_{4.759}$, and $RT_{6.072}$) decreased with the simultaneous appearance of new peaks ($RT_{5.325}$ and $RT_{5.273}$) after 30 and 120 min of co-incubation respectively. Further incubation led to the appearance of two new peaks ($RT_{5.568}$ and $RT_{5.851}$). New peak formed ($RT_{5.25}$) in the samples co-incubated for 24 h was disappeared in the sample co-incubated for 48 h and size of peak ($RT_{5.445}$) decreased with formation of two new peaks ($RT_{4.07}$ and $RT_{4.35}$). These changes in the HPLC chromatogram indicate extensive degradation of MeIQx (Fig. 6).



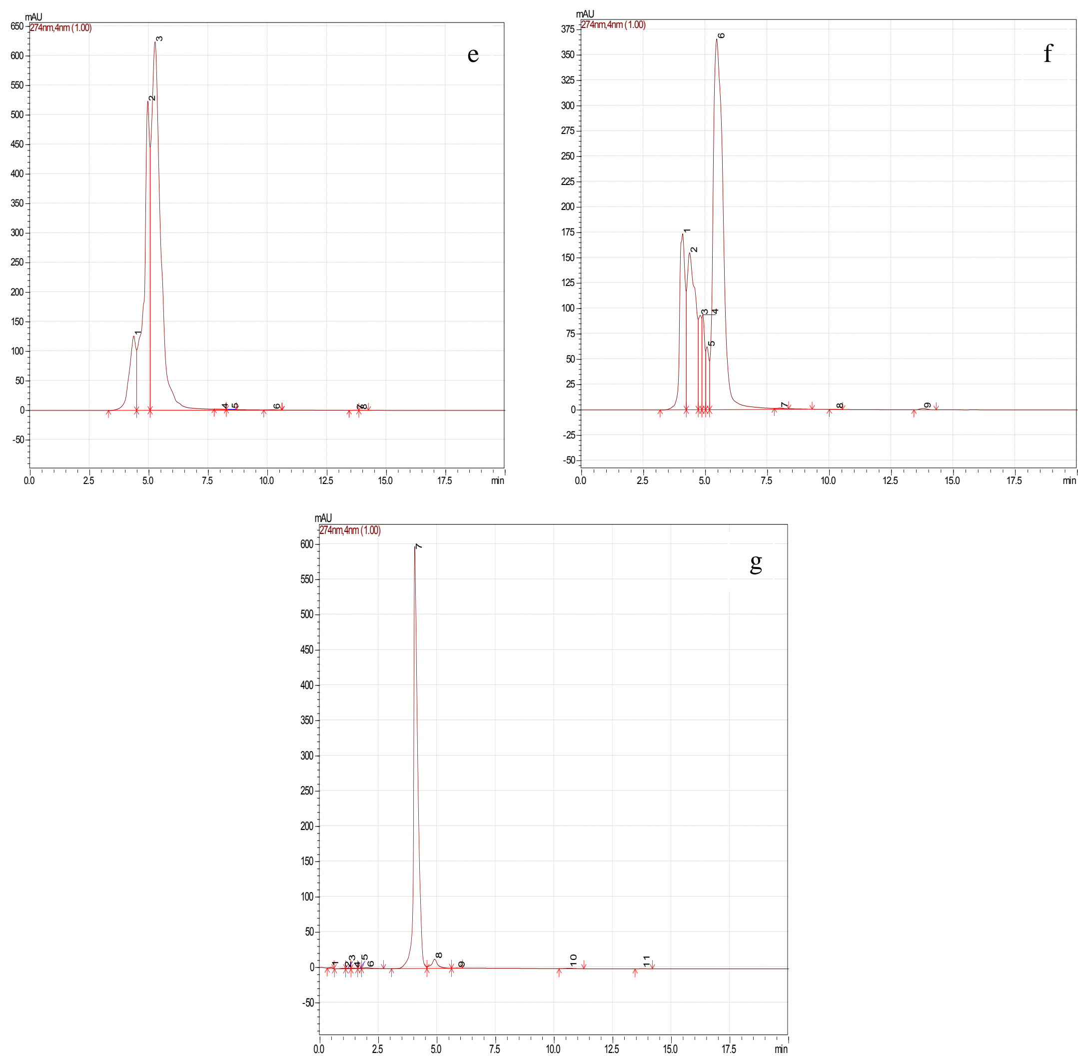


Fig. 6 HPLC chromatogram of supernatants of co-incubation assay mixture containing MeIQx and cells of *L. rhamnosus* Fb analyzed at different time intervals

[a] control MeIQx (RT$_{5.581\ \mathrm{min}}$)

[b] 30 min (RT$_{5.325}$ min)

[c] 120 min (RT$_{5.273\ \mathrm{min}}$)

[d] 180 min (RT$_{5.568\ \mathrm{min}}$, and RT$_{5.851\mathrm{min}}$)

[e] 24 h (RT$_{5.25\ \mathrm{min}}$)

[f] 48 h (RT$_{5.445\ \mathrm{min}}$, RT$_{4.07\ \mathrm{min}}$, RT$_{4.35\ \mathrm{min}}$)

[g] Growth control without MeIQx (RT$_{4.039}$ min)

Discussion

In this study, we have evaluated human origin *Lactobacillus* strains capable of adsorbing and biotransforming the food-borne carcinogen MeIQx. MeIQx is a heterocyclic amine prominent in protein rich foods, which is formed at high temperature; and its concentration ranges between ing 1-89 ng/g protein rich food (Layton *et al*., 1995; Sinha *et al*., 1998). It was estimated that the daily intake of HCA is from 1-17 ng/kg of the body weight (Layton *et al*., 1995). It is important to note that HCAs have been identified in beer, cigarette, wine, exhaust fumes, smoke and many other sites, so actual human exposure to the carcinogens is unknown (Yang *et al.*, 1998). Like many other environmental carcinogens, MeIQx requires metabolic activation to exert toxic effects.

Zhang *et al*. (1991) reported *Streptococcus cremoris* Z-25, *L. acidophilus* IFO 13951 and *B. bifidum* IFO 14252 bound 8.83, 5.37 and 1.72 µg/mg freeze-dried bacterial cells respectively. Zhang *et al.* (1993) reported cell wall skeleton of *L. acidophilus* IFO 13951 exhibited 9% reduction in mutagenicity and 10% binding. Orrhage *et al*. (1994) describe *L. acidophilus* NCFV 1748, *L. fermentum* KLD and *B. longum* BB536 to bind 20-40% MeIQx. Terahara *et al*. (1998) reported cells of *L. delbrueckii* subsp. *bulgaricus* 2038 and *S. thermophilus* 1131 bound MeIQx 60.8 and 32.3% respectively. Rajendra and Ohta (2001) reported lyophilized cells of *Bacillus natto* bound 58.6% and also showed that cell wall rather than cytoplasm played an important role in the pH-dependent binding of MeIQx. Stidl *et al*. (2008) postulated that LAB prevents genotoxic and/or carcinogenic effects of heterocyclic amines (AαC, PhIP, IQ, MeIQx and DiMeIQx) in laboratory rodents and humans *via* direct binding mechanisms. Nowak and Libudzisz (2009) reported *L. casei* DN114001 to bind MeIQx and reported 35-65% antigenotoxic activity and it depended on the incubation time, cell growth and medium used. With these previous findings, our study reports the *in vitro* binding ability of resting viable cells of *Lactobacillus rhamnosus* strains caused 58-63% adsorption of MeIQx and binding of MeIQx leads to the biotransformation of MeIQx. Biotransformation of MeIQx was evidenced in the UV spectral modifications characterized by spectral shift towards shorter wavelength, and decreased absorbance at the A_{342} and HPLC chromatogram. Decrease and the disappearance of the original peaks of MeIQx upon prolonged incubation observed in

HPLC chromatograms implicate binding and subsequent biotransformation and degradation of MeIQx.

MeIQx adsorption precedes biotransformation of MeIQx and is dependent on incubation time. MeIQx biotransformation occur higher in the pH range 7-10 which allows us to hypothesize that in the intestine where absorption of food is higher and pH is around 7 can favour the biotransformation of MeIQx. These results are in accordance with the previous findings by Orrhage *et al*. (1994) reported the binding of *L. acidophilus* to Trp-P-2 was less effective under acidic conditions (pH 3.0). Simulated gastrointestinal fluid treated cells notably adsorbed the MeIQx, though biotransformation ability decreased. The current experiments were carried out with an aim to mimic the human gastrointestinal tract.

Pretreated cells with HCl, $MgCl_2$, $CaCl_2$, $NaIO_4$ and TCA causes inhibition of MeIQx biotransformation, which is influenced by the carbohydrate components of the cells. Protein-denaturing treatments such as NaOH, SDS and LiCl also inhibited MeIQx biotransformation. Moreover, the surface associated protein extracts cause MeIQx biotransformation while LiCl-treated cells exhibit decreased MeIQx biotransformation activity implicating the importance of surface layer proteins in MeIQx biotransformation. Inhibition of MeIQx biotransformation with urea-treated cells shows importance of hydrophobic interactions in MeIQx biotransformation. Variously treated cells cause the inhibition of binding and biotransformation of MeIQx, suggesting the role of multiple components in MeIQx biotransformation.

Overall, experimental evidences demonstrate that *Lactobacillus* strains bind and biotransform food-borne heterocyclic amine MeIQx but also extensively degrade MeIQx. The results give rise to the possibility of increasing the number of bacterial cells in the human gut that would presumably decrease mutagens bioavailability and thus prevent absorption by colon epithelium. Moreover, peptidoglycan and the cell wall carbohydrates and proteins form a part of the receptors facilitating the binding of MeIQx. Probiotic strains with such abilities to bind and biotransform MeIQx, would be beneficial. Further studies are in progress to determine the antigenotoxic and antimutagenic activities against MeIQx by these strains.