CHAPTER 3

In-vivo localization of Probiotic Escherichia coli containing Vitreoscilla hemoglobin (vgb) gene in rats and its effects in colonization

3.1 Introduction

Human intestine has a very complex microbiota, with approximately 500-1000 different species (Peekhaus and Conway, 1998). At birth, babies emerge from a sterile environment into one that is loaded with microbes as a result of which the infant's intestine rapidly becomes home to one of the densest populations of bacteria on Earth (Comstock *et al.*, 2007). Approximately ($\sim 10^{14}$) number of microorganisms are present in a normal healthy individual which is 10 fold greater than the total number of cells present in human body ($\sim 10^{13}$). The endogenous GI microbial flora plays a fundamentally important role in health and disease, yet this ecosystem remains to be incompletely characterized (Falk *et al.*, 1998; Backhed *et al.*, 2005). Critical functions of the commensal microflora include protection against irritable bowel syndrome, inflammatory bowel disease, colorectal cancer, epithelial cell injury, regulation of host fat storage, and stimulation of intestinal angiogenesis (Bik *et al.*, 2005; Stanghellini *et al.*, 2010).

In the GI tract the microflora diversity changes from stomach to rectum. Microbiota of infants possesses three taxonomic groups whereas healthy adults contain only two phyla (Bik *et al.*, 2005; Turnbaugh *et al.*, 2006; Comstock *et al.*, 2007). Amongst proteobacteria, *E. coli* is the predominant commensal microorganism present in the GI tract (Tenaillon *et al.*, 2010). *E. coli* being a facultative anaerobe colonizes GI tract at early stages and proposed to facilitate the colonization of obligate anaerobes belonging to 22 different phyla by creation of reduced environment (Palmer *et al.*, 2007). Many *E. coli* strains were demonstrated to have probiotic properties (Adler, 2006; Cursino *et al.*, 2006; Gillor *et al.*, 2008). Previously, we had isolated *E. coli* strains from rat feacal matter contained following characteristic (acid tolerent, antibiotic susceptibility, non-pathogenic and antimicrobial activity against the members of *Enterobacteriacae* family) which made it a good potential Probiotic (Kumar *et al.*, 2009).

Oxygen EPR imaging showed that the intestine fluctuates between anaerobic and microaerobic conditions **Fig. 3.1** (He *et al.*, 1999). To adapt to the microaerobic

environment of intestine bacteria needs a control over aerobic and anaerobic genes (Falk *et al.*, 1998). Facultative anaerobe *E. coli* has Aerobic Respiratory Control (ARC) system and Fumarate Nitrate Reductase (FNR) system for aerobic and anaerobic control **Fig. 3.2** (Tsai *et al.*, 1995).

Fig. 3.1 Change in oxygen tension at different levels of the GI tract (He *et al.*, 1999)

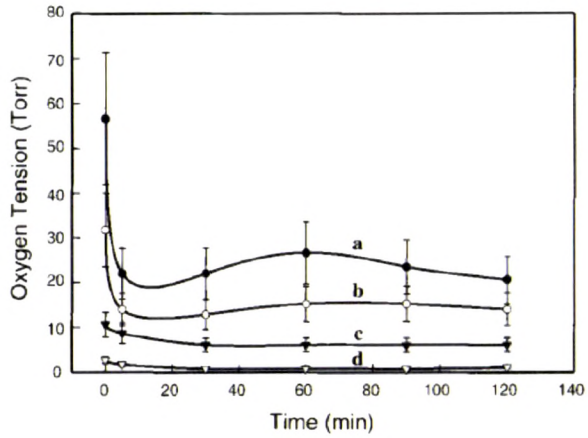
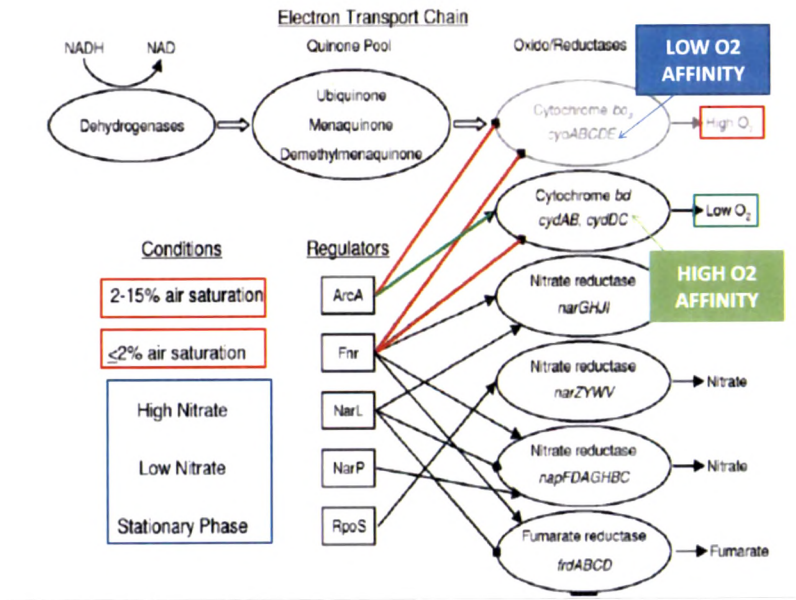
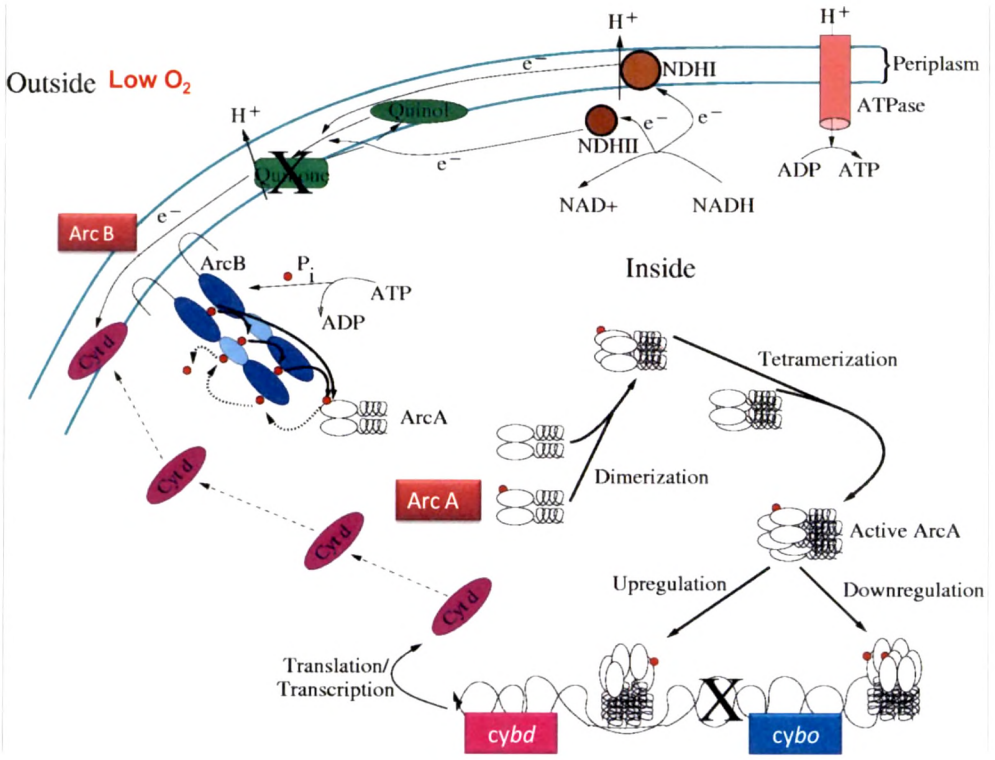


Fig. 3.2 Overview of mechanism of Arc/Fnr system (Jones *et al.*, 2007)



Under low outside O_2 tension, ArcB gets activated by auto phosphorylation using ATP as phosphate donar. Activated ArcB activates ArcA by transferring phosphate group to ArcA which in turn under goes tetramarization and become fully active. This activated ArcA tetramer suppresses *cyt bo* oxidase (low O_2 affinity cytochrome oxidase) and activates *cyt bd* oxidase (high O_2 affinity cytochrome oxidase). Thus now cell can respire though O_2 tension is below optimum **Fig 3.3** (Peekhaus and Conway, 1998).

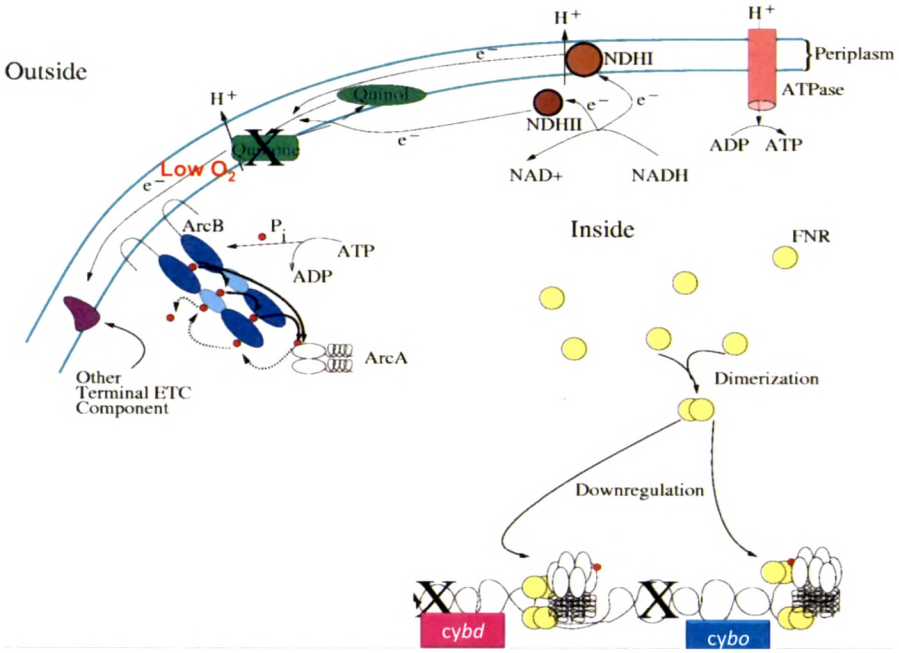
Fig. 3.3 Mechanism of ARC system (Peekhaus and Conway, 1998)



When O_2 tension is below 2% saturation, Fnr gets activated by autophosphorylation and under goes dimerization. This dimerized activated Fnr suppresses expression of Cyt bo oxidase (low O_2 affinity cytochrome oxidase) and Cyt bd oxidase (high O_2 affinity cytochrome oxidase). Thus the cells are dependent on nitrate respiration when Fnr is

active. When no exogenous addition of any electron acceptor is there then fermentation is major ATP source for organism **Fig 3.4** (Peekhaus and Conway, 1998). Previous studies reported that aerobic bacterial respiration is essential for effective competition and colonization of *E. coli* in microaerobic environment of intestine (Jones *et al.*, 2007).

Fig. 3.4 Mechanism of FNR system (Peekhaus and Conway, 1998)



Expression of *vgb* gene increases the effective intracellular oxygen concentration under microaerobic conditions, and improves growth of *E. coli* under oxygen-limited conditions. The natural promoter of *vgb* gene is oxygen sensitive promoter with ArcA binding site, which regulates VHb expression positively. In oxygen poor habitats *Vitreoscilla* spp. *a* obligate aerobe survives due to efficient oxygen-binding kinetics of *Vitreoscilla* hemoglobin (*vgb*) gene which improves its productivity under hypoxic conditions (Kaur *et al.*, 2008) This regulation helps *vgb* gene to get expressed in

microaerobic condition. VHb has been shown to enhance cell density, oxidative metabolism, engineered product formation, and bioremediation, especially under oxygen-limiting conditions (Stark *et al.*, 1999; Erenler *et al.*, 2004). A heterologous expression of *vgb* improves the efficiency of microaerobic respiration and growth of *E. coli* under hypoxic condition (Tsai *et al.*, 1995). Similarily, Heterologous expression of *vgb* gene in *Enterobacter aerogenes* reduced H_2O_2 toxicity (Geckil *et al.*, 2003). VHb has also been shown to possess peroxidase activity (Kvist *et al.*, 2007; Ayudhya *et al.*, 2010). This protective role of VHb is mediated through oxidative stress regulator OxyR which in turn activates VHb biosynthesis (Anand *et al.*, 2010; Akbas *et al.*, 2011). VHb in *E. coli* induces the expression of *kat G* (catalase–peroxidase G) and *sod A* (superoxide dismutase A) genes, thereby, protects from the damage caused by reactive oxygen species. Chimera of SOD and VHb protein rapidly detoxified reactive oxygen species in *E. coli* (Isarankura-Na-Ayudhya *et al.*, 2010).

Previous studies reported that carbon tetrachloride causes tissue injury especially in hepatocytes by formation of highly reactive trichloromethyl radical *in vivo* condition (Natrajan *et al.*, 2006). Trichloromethyl radical reacts with molecular oxygen to form trichloromethylperoxyl radical and oxidizes lipids molecules by hydrogen abstraction especially in hepatocytes. In the present study, we investigated the effect of *vgb* gene expression on intestinal residence time of *E. coli* 16 and protection against chemically induced oxidative stress damage in rats.

3.2. Materials and methods

3.2.1 Bacterial strains, plasmids and culture conditions

The bacterial strains and the plasmids used in the present study are listed in **Table 3.1**. *E. coli* 16 was used throughout the study as these possess good probiotic properties and acid tolerance capability at pH- 2 (Kumar *et al.*, 2009). Isolates were maintained on Hichrome coliform agar and MacConkey agar plates (Himedia). *E. coli* DH5α was used for constructing recombinant plasmids. *E. coli* BL21 was used for expressing the proteins. Luria-Bertani (LB) rich medium [5 g/l yeast extract (Himedia), 10 g/l, Tryptone (Himedia), and 10 g/l NaCl] or Teriffic broth and M9 minimal medium (12.8 g/l $Na_2HPO_4 \cdot 7H_2O$, 3 g/l KH_2PO_4, 0.5 g/l NaCl, 1 g/l NH_4Cl, 3 mg/l $CaCl_2$, 1 mM $MgSO_4$) were used for plasmid construction and cell culture, respectively. $NaNO_3$ (10 g/l) was added to the medium for induction of the *nar* promoter and 1 mM $FeSO_4$ was added as a metal cofactor for VHb protein. Plasmid-containing cells were grown in medium supplemented with 100 μg/ml ampicillin.

3.2.2 Recombinant plasmids construction and transformation in *E. coli* 16.

Green fluorescent protein (*gfp*) gene along with modified *lac* promoter (obtained as a PvuII fragment of 1.1kb) from pUC18-*gfp* plasmid was incorporated into SmaI site of pUC8:16 plasmid to obtain pUC8:16-*gfp* plasmid. The recombinant plasmid was confirmed by restriction digestion. The plasmids pUC18-*gfp* and pUC8:16-*gfp* was independently transformed in the potential probiotic *E. coli* 16 using the $CaCl_2$ method (Sambrook *et al.*, 2001). The transformant colonies were screened by their fluorescent at 365nm in U.V. transilluminator.

Table 3.1: List of bacterial strains and plasmids used:

Plasmids/Strains	Relevant characteristics	Reference /Source
Plasmids		
pUC-*gfp*	derived from the high-copy number vector pUC18 by insertion of a modified *gfp* gene; Ap^r	Schultz *et al.* 2005
pUC8:16	derived from the high-copy number vector pUC8 by insertion of a *vgb* gene; Ap^r	Stark *et al.* 1994
pUC8:16-*gfp*	derived from the high-copy number vector pUC8:16 by insertion of a *gfp* gene; Ap^r	This study
Bacterial strains		
E. coli DH5α	*F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ–*	Sambrook and Russell. 2001
E. coli BL21	*F' ompT hsdSB (rB– mB–) gal dcm*	Sambrook and Russell. 2001
E. coli isolate16	Wild type	Kumar *et al.* 2009
*E. c.*16 (pUC-*gfp*)	*E. coli* isolate 16 with pUC-*gfp* plasmid; Ap^r	This study
*E. c.*16 (pUC8:16-*gfp*)	*E. coli* isolate 16 with pUC8:16-*gfp* plasmid; Ap^r	This study

3.2.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed to detect GFP and VHb protein. Samples were mixed with sample buffer [0.06 M Tris-HCl (pH 6.8), 10% glycerol, 2% SDS, 5% β-mercaptoethanol (Sigma), and 0.01% bromophenol blue (Sigma)], incubated at 100°C for 3 min, centrifuged briefly, and loaded onto a 12% slab gel. After electrophoresis, the gel was stained with

Coomassie blue (Sigma) and silver nitrate (Sambrook *et al.*, 2001). The stained gel was scanned, and the digitized image was stored and analyzed.

3.2.4 Preparation of cells and cell free extracts for catalase assays.

Luria broth grown cells were treated with CCl_4 (65mM) at 0.5 O.D i.e mid-log growth phase and kept for 30h and then harvested the cell culture by centrifugation 9,200 g for 2 min at 4°C. The cell pellet was washed once with 50 mM phosphate buffer (pH 7.0) followed by re-suspension in same buffer. The cells were then subjected to sonication (Branson Sonifier Model 450) for total period of 1 min at pulse rate of 30 s in an ice bath, followed by centrifugation at 9,200 g at 4°C for 30 min to remove cell debris. The supernatant was used as cell-free extract for the catalase assays.

3.2.5 Catalase assay of cell free extract.

Cell free extract prepared as above, was added in a cuvette along with 30mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.0), and the decrease in absorbance was measured at 240 nm for 1 min to determine catalase activity (Aebi, 1984). The molar extinction coefficient of 43.6 M/cm was used to determine catalase activity and reported in units/min/mg of protein.

3.2.6 Animal Experiments

3.2.6.1 Experimental animals: Male Charles foster rats were housed in the departmental animal house under controlled room temperature (21 ± 2 °C). The animals were provided with rat chow and water *ad libitum.* The experiments were carried out after the approval of Animal Ethical Committee of Department of Biochemistry, The M.S. University of Baroda, Vadodara (Approval No. 938/A/06/ CPCSEA), and CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines were followed.

3.2.6.2 Colonization experiments.

2-3 month of age rats, were given drinking water containing streptomycin sulfate (5 g/liter) for 24 h to remove the existing resident facultative microflora and then starved for food and water for 18-20 h. The rats were divided into two groups and were fed approximately 10^9 CFU of *E. coli* 16 pUC-*gfp* and *E. coli* 16 pUC8-16*gfp* in 1 ml of 20% sucrose for regular three days respectively. After the bacterial suspension was ingested, food and water were restored, feacal plate counts were determined at regular interval till 70^{th} days. Feacal samples were homogenized, serially diluted in 0.85% saline and plated on Luria agar plate containing ampicillin (100µg/ml). After 24 h, plates were inspected under UV light. As soon as the reduction of fluorescent colonies from feacal samples was noted (day 23 and day 48), rats were given ampicillin (50 mg/kg body weight) (days 23-25 and days 48-51) in drinking water, followed by culture of the feacal flora (Schultza *et al.*, 2005; Jones *et al.*, 2007).

3.2.6.3 Animal study to monitor effect of *E. coli* 16 pUC8-16*gfp* under oxidative stress.

A total of 15 rats (14 to 16 months) were equally divided into 5 groups (n = 3) Group I served as normal control and was orally given saline for 3 days and then biweekly interval for 45 days. Group II served as probiotic *E. coli* 16 pUC8-16*gfp* plasmid and culture was orally given along with saline for 3 days and then biweekly interval for 45 days. Group III (normal control with CCl_4) were orally given saline same as group I and after 45 day, two doses of CCl_4 200µl and 500 µl was given along with olive oil as carriers at weekly interval and monitored the antioxidant parameter in plasma and liver to assess the liver function. Group IV (probiotic *E. coli* 16 pUC-*gfp* with CCl_4) served as vector control and same procedure was done as group III. Group V (probiotic *E. coli* 16 pUC8-16*gfp* with CCl_4) served as test and same procedure was done as Group III. At the end of the 2^{nd} dose, on the third day rats were mildly anaesthetized and blood was collected via retro-orbital sinus and plasma was separated for further biochemical

analysis. Later, animals were sacrificed by decapitation under mild anaesthesia and liver was excised and stored at -80^0 C for further estimations.

3.2.6.3.1 Assessment of liver function

Serum glutamic pyruvate transaminase (SGPT) and Serumglutamic oxaloacetic transaminase (SGOT) were assayed in plasma sample using commercially available kits (Reckon diagnostics).

3.2.6.3.2 Hepatic lipid peroxidation and catalase assay.

Samples of liver (100 mg/ml) were homogenized in 50mM potassium phosphate buffer and centrifuged at 10,000 rpm for 15 min and the supernatant thus obtained was used for biochemical analysis. All parameters were expressed as activity per mg of protein. The protein concentration in each fraction was determined by modified Lowry using bovine serum albumin as standard. The mean malondialdehyde (MDA) content (μmol/mg protein), a measure of lipid peroxidation, was assayed in the form of thiobarbituric acid-reacting substances (TABRS) by the method of Ohkawa *et al.*, 1979. Catalase assay was measured by the method described by Aebi *et al.*, (1984).

3.2.6.3.3 Microscopic examination of liver.

Liver samples were fixed in 4% buffered paraformaldehyde, dehydrated in graded alcohol series and embedded in paraffin wax. About 4-5-mm thick sections were cut (by Leica RM 2155 Microtome) and stained with hematoxylin and eosin and examined under Leica microscope.

3.2.7 Statistical analysis

Statistical evaluation of the data was done by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test and results were expressed as mean ± SEM using Graph Pad Prism for Windows, Graph Pad Software, San Diego, California, USA.

3.3 Results

3.3.1 *In vitro* studies

3.3.1.1 Growth profile and SDS PAGE of *E. coli* isolate 16 harboring GFP and VHb-GFP under microaerophilic condition.

To monitor the *E. coli* isolate no 16, the *gfp* gene from pUC-*gfp* plasmid was tagged along with *vgb* under the control of *lac* promoter generating pUC8-16*gfp* vector. Under microaerophilic condition, the probiotic *E. coli* 16 pUC8-16-*gfp* transformants had significant increase in growth rate ($P < 0.01$) as compared to their pUC*gfp* as a control vector (**Fig.3.5**). SDS-PAGE profile in the aerobic condition did not show the presence of VHb protein but it was detected under microaerobic condition (**Fig. 3.6A and B**).

Fig. 3.5. Growth curves of *E. coli* 16 pUC*gfp* and pUC8-16*gfp* transformants

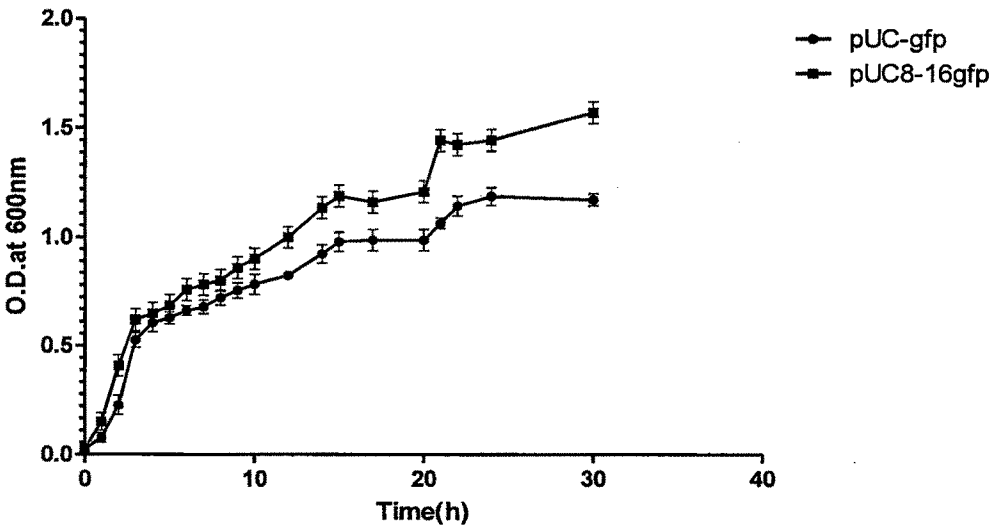


Fig. 3. 6A. SDS-PAGE analysis of *E. coli* BL21 pUC8-16*gfp* lysate under aerobic condition

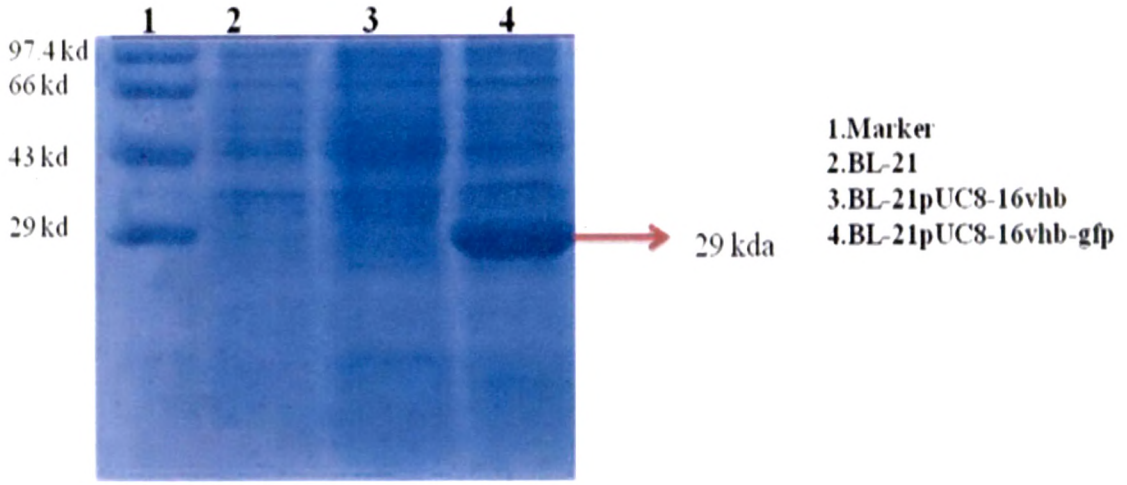
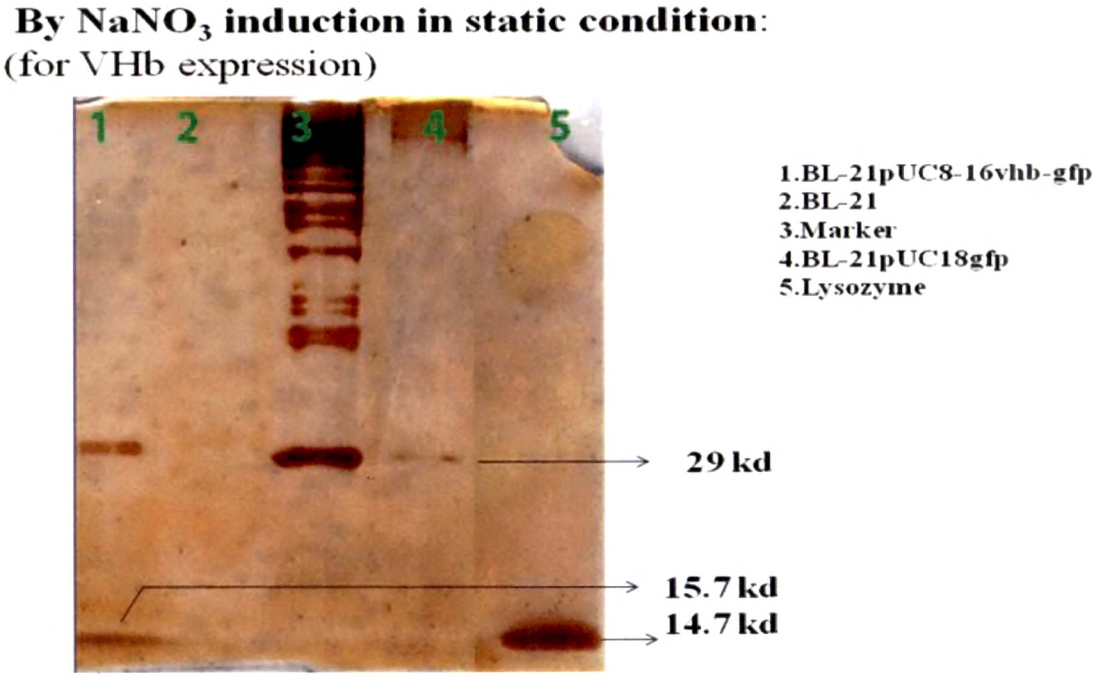


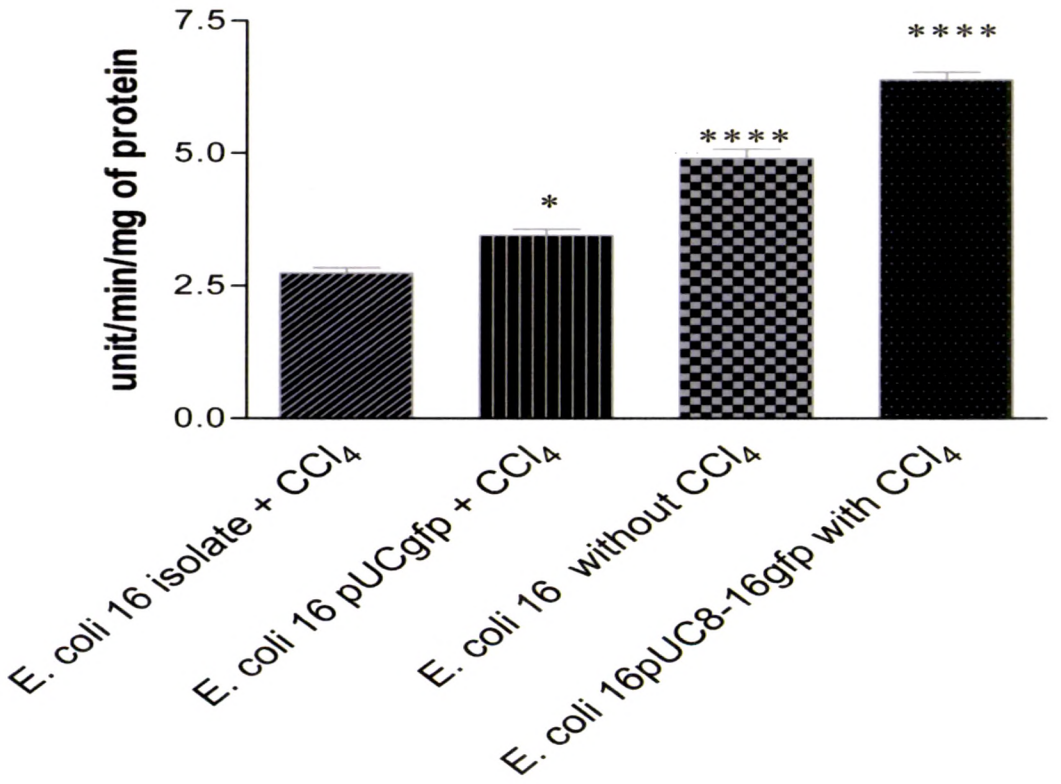
Fig. 3.6B. SDS-PAGE analysis for expression of *E. coli* BL21 pUC8-16*gfp* by $NaNO_3$ induction condition.



3.3.1.2 VHb enhances *in vitro* catalase activity of *E. coli* 16 expressing VHb protein.

As VHb protein known to causes catalytic destruction of hydrogen peroxide like catalase enzyme under oxidative stress condition (Kvist *et al.*, 2007) we tested *in-vitro* an antioxidant activity of VHb in *E. coli* 16 harboring pUC8-16*gfp* plasmid. *In vitro* antioxidant activity of probiotic *E. coli* 16 harboring pUC8-16*gfp* plasmid was monitored by the catalase enzyme activity under chemical induced oxidative stress condition. In presence of CCl_4, catalase activity in *E. coli* isolates 16 containing *vgb* gene was increased by 1.8 fold as compared to control *E. coli* isolate 16 with only *gfp* gene (**Fig. 3.7**). This suggests that VHb is expressed under microaerophilic environment and is functional when expressed in *E. coli* isolates 16.

Fig.3.7. *In vitro* catalase activity of potential probiotic *E. coli* isolates containing pUC8-16*gfp* plasmid



3.3.2 *In vivo* studies

3.3.2.1 Colonization of probiotic *E. coli* 16 *vgb-gfp* in gastro-intestinal tract of rats exposed with intermittent antibiotic challenge.

The colonization of Charles Foster rats by *E. coli* 16 isolates transformed with pUC8-16*gfp* and pUC-*gfp* plasmid independently were monitored after 3-day oral antibiotic pre-treatment with streptomycin followed by a 3-day period of oral administration of 10^9 CFU of *E coli* 16 (pUC8-16*gfp*) and *E. coli* 16 (pUC-*gfp*) transformant. *E. coli* 16 (pUC-*gfp*) numbers was declined in feces significantly compared to *E coli* 16 (pUC8-16*gfp*) transformants. On 21^{st} day, the feacal cultures for *E. coli* 16 (pUC-*gfp*) were 100 times reduced compared to *E coli* 16 (pUC8-16*gfp*). After 22^{nd} to 24^{th} days, the first treatment of ampicillin was given which increased the numbers of both *E coli* 16 (pUC-*gfp*) and *E. coli* 16 (pUC8-16*gfp*) transformants in feces. After ampicillin treatment on 48 days *E. coli* 16 (pUC- *gfp*) was not detected but *E. coli* 16 (pUC8-16*gfp*) remained stable even after second ampicillin treatment upto 70^{th} day (**Fig 3.8A**). Thus, the residence time of probiotic *E. coli* 16 pUC8-16*gfp* was significantly improved in gastro intestinal tract of rats. On 48^{th} days post feeding (**Fig.3.8 B and C**), cultures from the feacal matter was spreaded on luria agar media, and checked for antimicrobial activity, we found fluorescence and antimicrobial property retained in *E. coli* harboring the pUC8-16*gfp* plasmid.

Fig 3.8 A. Feacal counts of *E. coli* 16 containing pUC-*gfp* and pUC8-16*gfp* plasmid transformants.

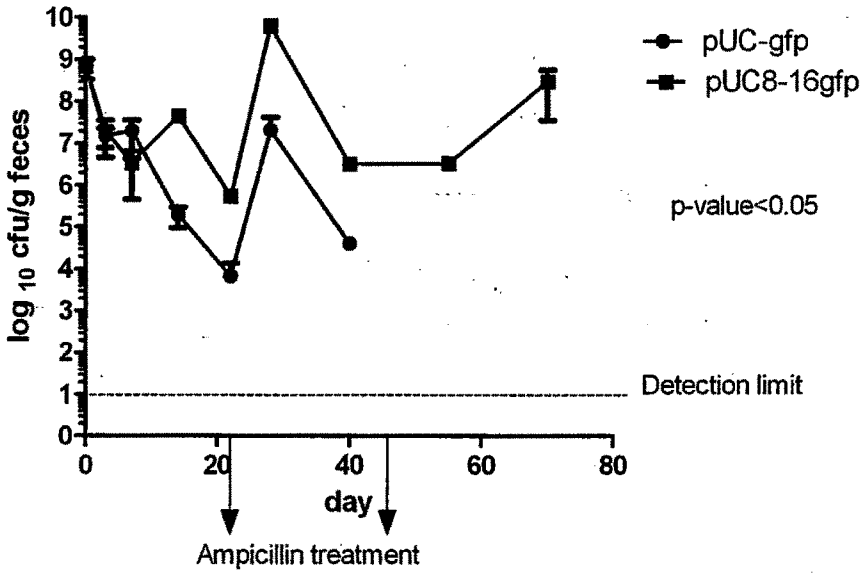


Fig. 3.8 B. Antimicrobial activity of *E. coli* 16 transformed with pUC8-16*gfp* plasmid in feacal samples of Charles Foster rats

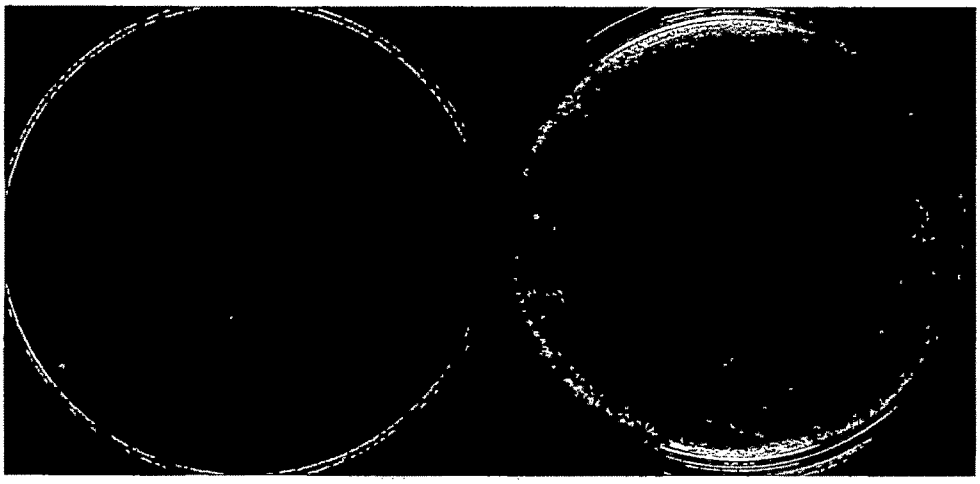
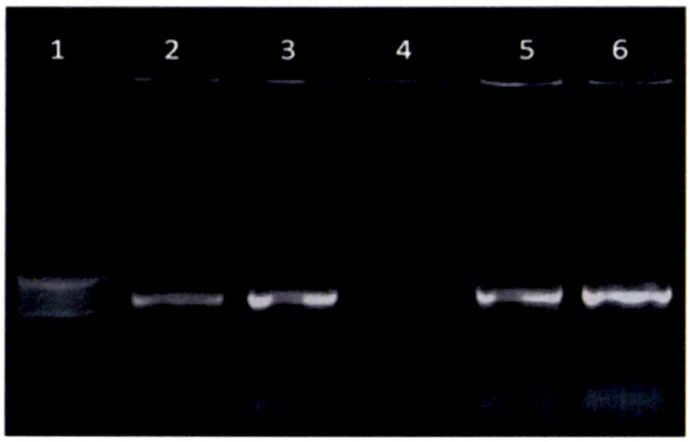


Fig. 3.8 C. Colony PCR amplification of *vgb* gene.



Lane 1: 100bp ladder

Lane 2: 714 bp

Lane 3 5: 714 bp

Lane 4: negative control

Lane 5: 714 bp

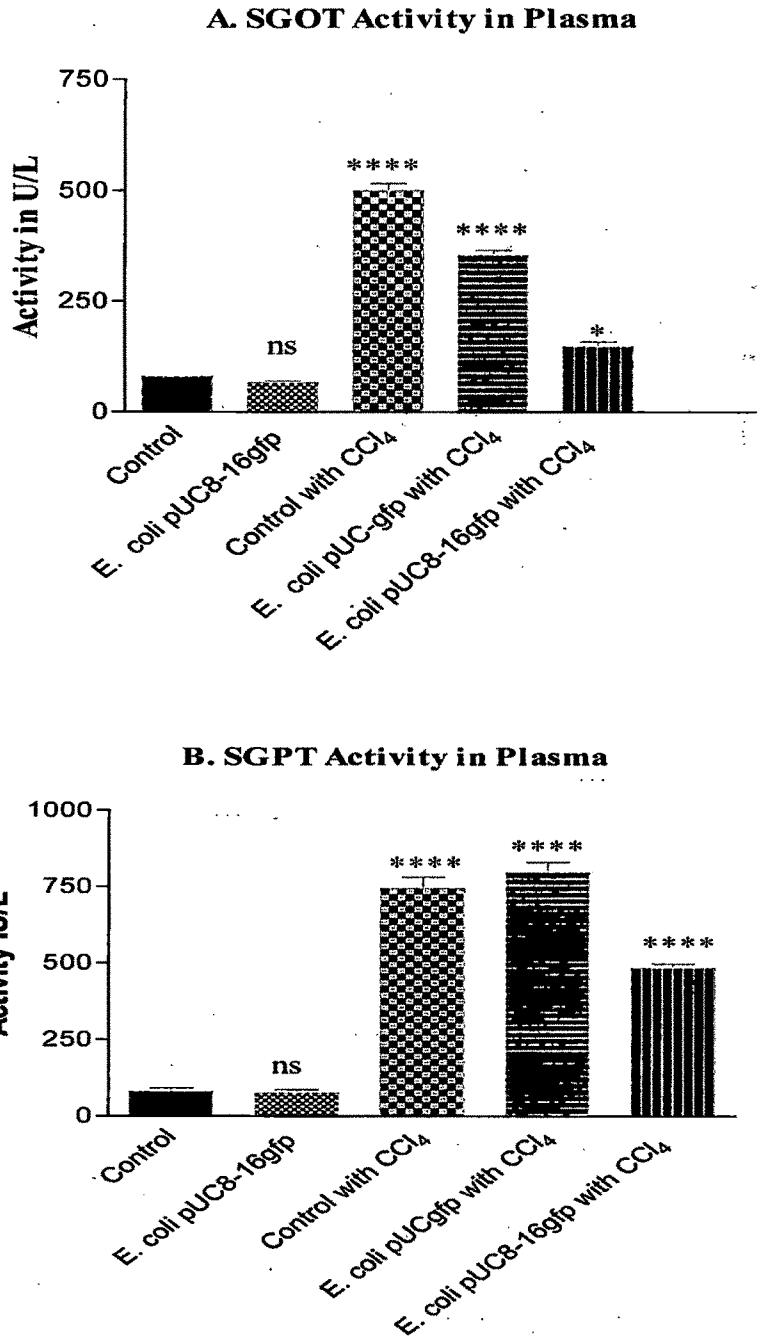
Lane 6: plasmid as positive control showed PCR.

3.3.2.2 Effects of probiotic *E. coli* 16 (pUC8-16*gfp*) harboring *vgb* gene on liver function under CCl_4 induced oxidative stress.

3.3.2.2.1 SGOT and SGPT activity in plasma sample of rats

VHb protein expression enhances *in vitro* catalase activity, thus its effect on **CCl_4** induced hepatotoxicity was investigated in Charles foster male rats under oxidative stress condition. Oral administration of 200 and 500 (µl/kg) of CCL_4 to rats at weekly interval resulted in significantly elevated ($p < 0.001$) SGPT and SGOT compared to control (Group I) and vector control (Group II) untreated rats. Exposure of CCl_4 to rats pre-fed with probiotic *E. coli* 16 pUC8-16*gfp* (Group V), the activities of SGOT and SGPT enzymes were significantly lower ($p < 0.05$) compared to rats of (Group III) and (Group IV) **Fig. 3.9A and B**. Rats with CCl_4-induced hepatotoxicity were pretreated with probiotic *E. coli* 16 pUC8-16*gfp*, serum SGPT and SGOT levels reverted to near normal.

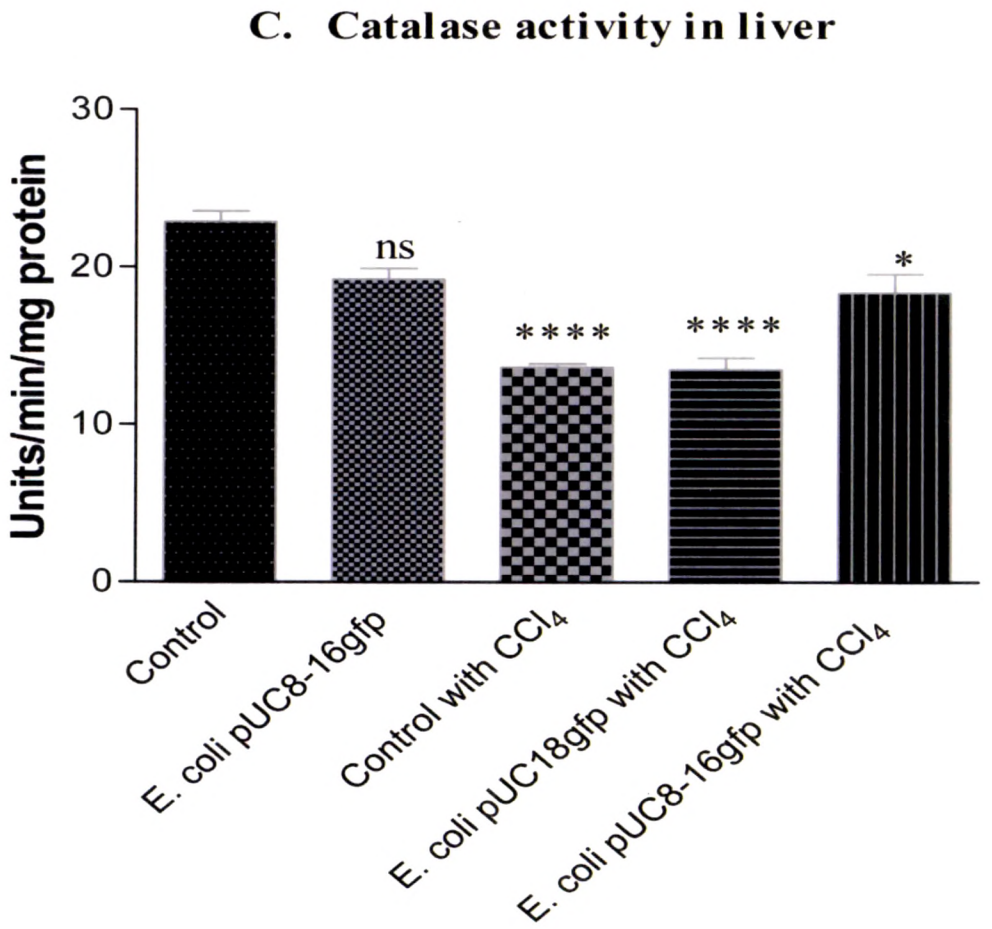
Fig. 3.9 A and B. SGOT and SGPT activitiy in plasma sample of rats.

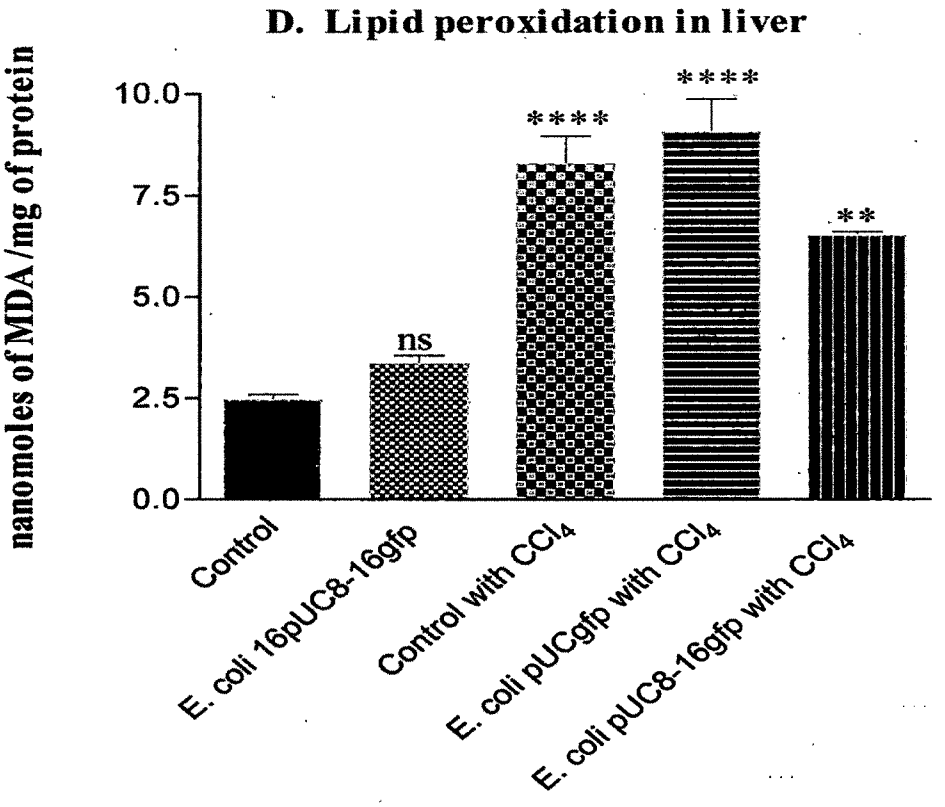


Values are expressed as mean ± SD of three rats in each group. $P < 0.05$ (ANOVA).

3.3.2.2.2. Catalase and lipid peroxidation activity in liver.

Catalase activity was significantly decreased in the liver of CCl_4 treated Group III and Group IV as compared to control groups. Probiotic *E. coli* pUC8-16*gfp* (Group V) showed significantly ($p < 0.05$) higher catalase activity as compared to CCl_4 treated Groups III (**Fig. 3.9C**). A Slight decrease in the mean MDA level was found in the liver of Group V (CCl_4-exposed) rats relative to Group III rats (**Fig. 3.9D**).

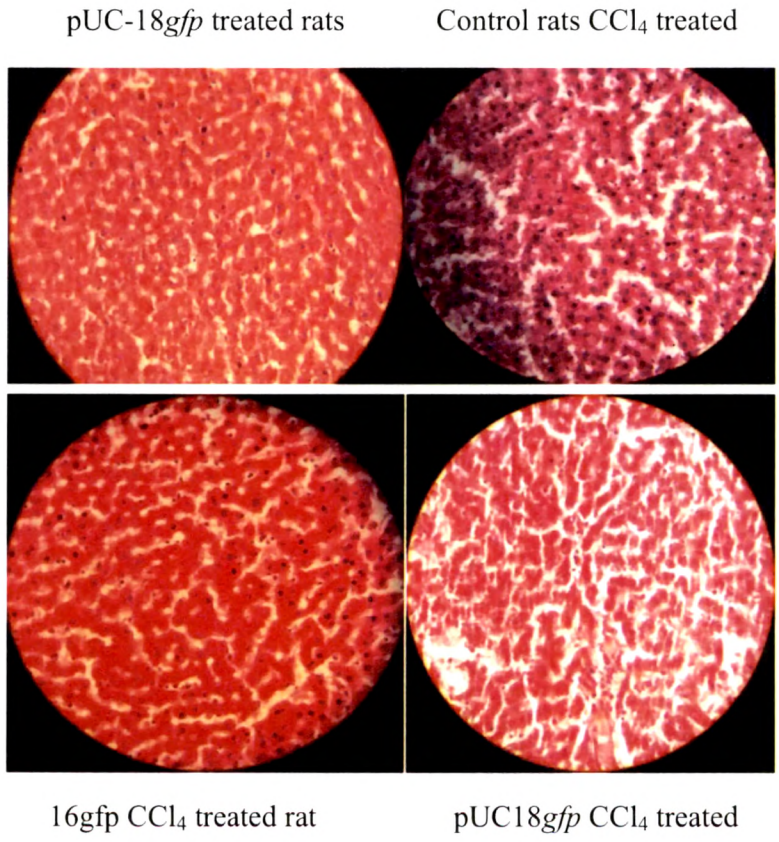




3.3.2.2.3 Microscopic examination of liver.

Histopathological analysis using hematoxylin and eosin stains of liver cells of Group III and Group IV rats (exposed to CCl_4) revealed extensive damage, characterized by the disruption of the lattice nature of the hepatocyte, damaged cell membranes, degenerated nuclei, disintegrated central vein and damaged hepatic sinusoids when compared to the liver of Group I & II (normal) animals. However, Group V rats (exposed to CCl_4 and pretreated with probiotic *E. coli* 16pUC8-16*gfp*), only minor disruption of the hepatic cellular structure was observed (**Fig. 3.10**).

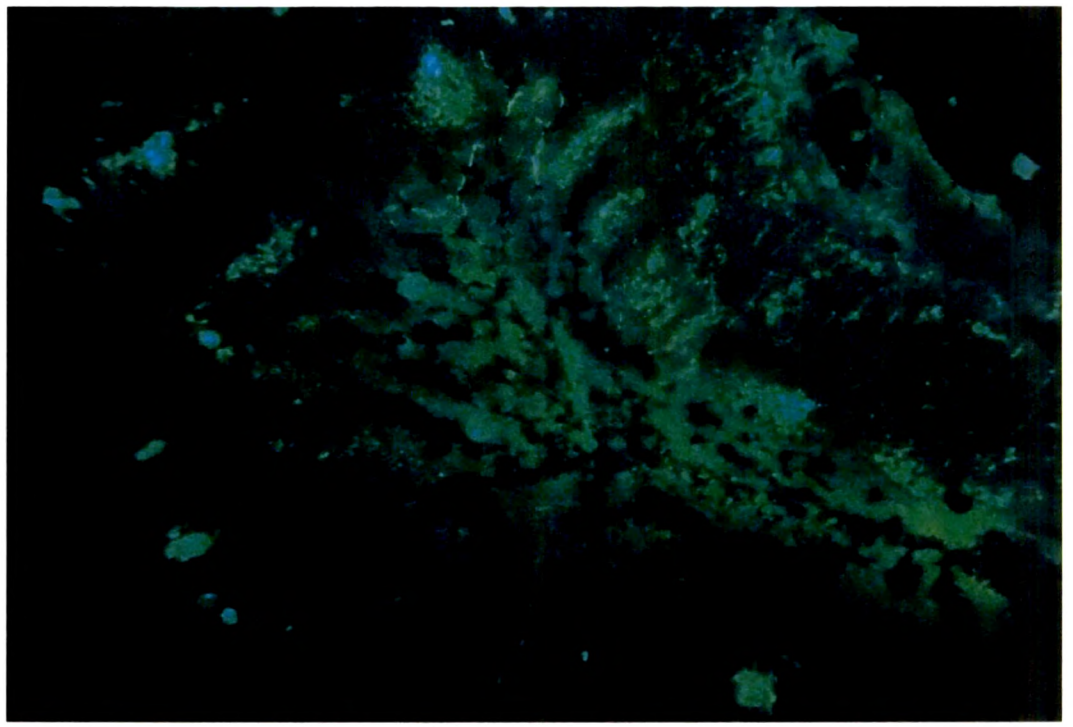
Fig. 3.10 Effect of probiotic *E. coli* harboring *vgb* gene on CCL_4-induced histopathological changes in rat liver. (A) Photomicrograph of liver from control rat with probiotic *E. coli* (pUC8-16*gfp*), (B) photomicrograph of liver treated with CCL_4, (C) photomicrograph of liver treated with CCL_4 along with probiotic *E. coli* (pUC8-16*gfp*) treated, (D) photomicrograph of liver treated with CCL_4 along with probiotic *E. coli* (pUC-*gfp*) treated. Haematoxylin and eosin staining of paraffin-embedded sections



3.3.2.2.4 *In vivo* localization of the *E. coli* 16 tagged with *gfp*

Transverse sections of the small intestine showed *gfp* tagged bacteria in patches adhering to the mucosa (**Fig. 3.11**). Washings from intestinal segments (approximately 2-4 cm) also showed presence of *gfp* tagged bacteria. The result shows that *E. coli* isolate no 16 has colonized better in the rat small intestine. Together these experiments suggest that the isolates were able to survive and colonize in the rat intestine. Further antimicrobial assay testing done with the labeled isolate obtained from the intestine showed that the isolate retains its antimicrobial activity even after passing through the GI tract.

Fig. 3.11 Fluorescence microscopy of histological sections of the small intestine of rats challenged with *gfp* tagged *E. coli* isolate 16. Colonized *gfp* tagged isolate appears as bright patches under fluorescence microscope



3.3.4 Discussion

Probiotic bacteria exert their effects by competing with potentially pathogenic bacteria for ecological niches, thereby preventing their colonization. Oxygen tension in the intestine may fluctuate due to dynamic cycles of oxygen diffusion and respiratory consumption by facultative anaerobes. What was the pathway operating for sugar utilization by *E. coli* in intestine is yet not understand but it is clear that Enter Doudoroff pathway is operating for gluconate utilization. Gluconate as a sole carbon source needed for successful colonization. The exact mechanism of colonization of *E. coli* in intestine is not clear, but it is known that respiration of *E. coli* in intestine is very much essential for its successful colonization and competitiveness in intestine (Jones *et al.*, 2007). Colonization and competitiveness of facultative anaerobes, i.e. *E. coli*, depends on their respiratory flexibility which in turn depends on high-affinity cytochrome bd oxidase. VHb improved oxygen uptake rate of *E. coli* under microaerobic condition, by 5 fold and 1.5 increase of cytochrome bo_3 and cytochrome bd oxidase, respectively (Tsai *et al.*, 1995a).

The expression of VHb protein under microaerophilic condition improves cell growth, protein synthesis, metabolite productivity and nitric oxide detoxification (Tsai *et al.*, 1995b; Geckil *et al.*, 2003; Isarankura-Na-Ayudhya *et al.*, 2008). The expression of VHb significantly improved colonization of probiotic *E.coli*16 harboring pUC8-16*gfp* plasmid in rat GI tract possibly due to improves cell growth and better respiratory adaptation under low oxygen tension.

Super oxide radical (O_2^-) formed within biological systems acts as a toxin to living cells. CCl_4 is well known as a hepatotoxin and generates oxidative stress in the intestine. $CCl_3OO^\cdot$ formed from O_2^- and CCl_4 have high toxic effect on metabolic oxidizing activities presumably because of the electron-withdrawing nature of the trichloromethyl group (Yamamoto *et al.*, 1998; Natarajan *et al.*, 2006). Heterologous expression of non-haem catalase in *Lactobacillus casei* improved the

antioxidant status and alleviated the risk of 1, 2 Dimethyl hydrazine induced colon cancer (Rochat *et al.*, 2006; LeBlanc *et al.*, 2008). Near to normal levels of SGPT and SGOT activity in CCl_4 treated rats with *E. coli* 16 (pUC8-16*gfp*) plasmid demonstrates the protection of the toxic effects in liver. The protective effects could be attributed to the peroxidase activity of VHb (Kvist *et al.*, 2007; Suwanwong *et al.*, 2006). VHb is known to decrease the oxidative stress of H_2O_2 by enhancing the catalase activity (Geckil *et al.*, 2003). VHb in *E. coli* induces the expression of *kat* G (catalase–peroxidase G) and *sod A* (superoxide dismutase A) genes, thereby, protects from damage caused by reactive oxygen species (Kvist et al., 2007). In comparison, when *vgb* gene was expressed in an *E. coli oxyR* mutant, *vgb* expression increased but the strain showed high sensitivity to oxidative stress without induction of antioxidant genes. Thus, oxidative stress regulator OxyR mediates the protective effect of *vgb* under oxidative stress (Anand *et al.*, 2010).

The present study demonstrated that v*gb* gene when expressed in a probiotic strain increases its residence time and improves its survival in GI tract and being an antioxidant it provides benefits to the organism against oxidative stress. As the residence time of probiotics is GI tract increases it reduces the doses to maintain effective probiotics count. These additional benefits may increase the efficiency of the probiotics making them more effective and also reduce their doses interval.