Metabolic Engineering of *Escherichia coli* as Probiotics

A THESIS SUBMITTED TO

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA

FOR THE DEGREE OF

DOCTORAL OF PHILOSOPHY IN BIOCHEMISTRY

BY

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January 2012

STATEMENT UNDER O. Ph.D. 8/ (iii) OF
THE M.S. UNIVERSITY OF BARODA, VADODARA

This is to certify that the work presented in this thesis is original and was obtained from the studies undertaken by me under the supervision of **Dr. G. Naresh Kumar.**

Vadodara — Prasant Kumar
Date: — Candidate

This is to certify that the work presented in the form of a thesis in fulfillment of the requirement for the award of the Ph. D. degree of The M. S. University of Baroda by Prasant Kumar is her original work. The entire research work and the thesis have been built up under my supervision.

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ACKNOWLEDGEMENTS

Finally the journey towards achieving something great is coming to its destination the most awaited moment which ultimately end up with the submission of my thesis; today when I look back I just think how myself able to make it a success. Yes myself alone may not have been able to achieve this but it is with the incessant efforts and support of numerous people, that I could arrive at my destination successfully and satisfactorily. At this juncture, I would like to take the privilege of acknowledging all those without whose help and support this journey would have been an impossible task. As I write these lines, the names and face of several people come to mind, who have contributed intellectually to this work, as well as to my overall growth and inclination towards science.

I am indebted to my research supervisor ***Prof. G.Naresh Kumar****(NK Sir), for his precious guidance; scientific counseling; never-ending patience, optimistic approach and powerful words of encouragement. His endless zest for science always has provided a positive thrust while the tea-time discussions with him on any-and-every topic on this earth, hardly made us feel like his students. I am extremely grateful to him for giving me an opportunity to learn, think and work in a liberal environment at Molecular Microbial Biochemistry Lab (MMBL). A practical lesson that always came from him when he said "You are not here to change the system; indeed make the most of the existing system and push ahead" had almost everything to explain if understood. His never ending enthusiasm , ever ready to take any kind of challenge and excellent management capacity really taught me a lot. If I put all the adjectives from dictionary still I cannot end writing about him.. Thanks a Ton Sir!*

I extend my heartfelt thanks to ***Dr. G. Archana****, Department of Microbiology and Biotechnology Centre, for her critical comments and for allowing me to use the facility of her lab (DGGE) whenever I approached which helped adding a different meaning to my research work. Thanks a lot for always being there to support Above all, I always relished the delicious food served with a flavor of warm hospitality.*

I would like to express my deep gratitude to ***Prof.B.B. Chattoo*** *and* ***Dr. Sanjya Jha (GRC)*** *who really made me compatible in planning and smooth handling of scientific experiments during my MSc. days.*

I am sincerely grateful to ***Prof. Rasheedunnisa Begum****, for allowing me to use her lab facilities anytime and for her constant encouragement to sincerely do good and competitive work.*

Special Thanks to ***Prof. Sarita Gupta*** *for her timely guidance and critical comments during my annual presentations which really helped me to refine my data.*

DR. S. R. Acharya*. deserves an extraordinary mention who have been always a source of inspiration to me. Thank you Sir for all the healthy scientific discussions and company for tea at DD.*

I am extremely grateful to all other faculty members; ***Dr. Pushpa Robin , Dr. C. RatnaPrabha ,Dr. Jayshree Pohnerkar, Prof. Tara Mehta,*** *for their encouraging words and criticism,valuable suggestions throughout the research tenure.*

Dr. LaxmiPriya and Prof.(Lt.) S.D. Telang *has been special figures during these years, whom I encountered during the initial period and helped me a lot in designing experiments,. I will always admire and remember LP mam for her pragmatic approach under any given situation.*

My heartiest thanks and gratitute to all the office staff including ***Mr. Pethe, Akshitaben, Shaileshbhai, Manishbhai , Rameshbhai (Nare) , Vyas bhai, Sandeep*** *and other non teaching staff members for their standing help and guidance in all the official matters.*

Special thanks ***Rameshbhai, Anilbhai, Narayan*** *whose extreme care of Animal House made the animal experiment to carry out successfully.*

Thankyou so much ***Bhartiben****, for helping during ordering of lab equipments and processing of all official accounts.Thanks to* ***Anilbhai*** *for helping me out with lab maintenance and make it an organized place to work in.*

I specially thank ***Mr. Jeetendra Dave, Bavin bhai*** *from Gujarat Chemicals for patiently listening to our sudden but urgent demands for chemicals/fine-chemicals and trying his levels best to deliver the order in time.*

I sincerely acknowledge the ***Indian Council of Medical Research (ICMR),*** *Government of India, for the JRF and SRF.*

I would like to thank Dr. HJ Linde for gifting the pUC 18-gfp, Dr Stark for pUC8-16plasmid and Dr Dijkhuizen for pET15b-inuj plasmid

I deeply acknowledge Prof. Santosh Kar and Atul pandey (JNU), for supported me in Cytokines Study .

Prof Joshi (veterinary Science,Anand) for valuable support in learning the technology and scientific advancement.

I extend my thanks to ***Prof. Krian Kalia*** *(Head, S.P.U) and Dr. Vikash for their support and gave me cooperation during winding up of my thesis work. I am also thankful to my senior faculty members (S.P.U) for their cooperation.*

Well! The MMBLites needs special attention. To start with. I acknowledge ***Dr. Vikash Sharma****, the senior-most research student when Iwas a freshie, who helped me get acquainted to the lab and*

learn to begin. ***Dr. Gopit Shah****,* ***Dr.Divya Patel****,* *Dr.* ***Aditi Buch, Dr. Jisha Elias and Dr. Prashant Kunjadia*** *have been great seniors, who helped me in different ways.*

Thanks to ***Mr. Hemanta Adhikary*** *for being supportive and encouraging all the time not only in the lab but also in day to day life.*

Mr. Sumit Pandey, Mr. Asish Singh *been simply been a good addition to the probiotic research group of MMBLs . Huge thanks both of you or the help and support.* ***Sonal madam and Praveena madam*** *have been sources of spiritual empowerment. Thanks to* ***Chanchal , Jitendra*** *and* ***Kavita*** *for their endless support.The aspiring neophytes* ***Archana, Ujwal*** *made a great company and I wish for the best in future research.*

It was a pleasure having seniors like ***Dr, Hemandra, Dr. Sajil M,Dr. Keyur Dave, Dr. Maulik Thakkar, Dr. Hiren Modi , Dr. Jyotika Rajawat, Dr. Heena Mir, Dr. Shirayu Pandya ,Dr Niraj Bhatt, Dr Pradeep and Dr. Prakash Pillai*** *who taught the dos and don'ts in research ever-helpful elderly advices. I have shared few of the most fun-filled moments with you all.*

Research was full of sudden planning of experiments but with the nextdoor- neighbours like ***Naresh , Mitesh*** *it was not a problem. The tension that "What if I run short of any reagent/resource and doubt in methodology for clinical protocol in the midst of the experiment?" never came to me. Thank you both! for being so supportive and collaborative.*

I am extremely thankful to, ***Mrinal, Nidheech*** *and* ***Rusikesh*** *for invariably unselfish help Whenever required as well as for making a wonderful and enjoyable company.*

Thanks to Mr. Sanket Soni (SGL) so called my same date resigstered batch mate and most probably same date we are submitting thesis. Any update of any product related to nutraceutical he knew information so even I talk after one week or one month he update me about any product launched in market.

I would like to applaud my dissertation students, ***Ferzin, Chintan, Anand, Jyoti, Ayush, Deepak*** *and* ***Sriram*** *for being a great helping hand all along and for creating a fun-filled work environment.*

I am thankful to one and all the research scholars, Department of Biochemistry viz.,Vijay,Purva, purna,Swapnali,Ankita,Akhilesh,Tusar,Anubha,Purva,Krisma,Chetan,Shohaib for their pleasurable company and I wish them all the very best.

Thanks to ***Chandra Prakash, (CP..ya) and Jagat*** *for their endless support till the end .Thanks to all research colleagues in Department of Microbiology and Biotechnology Centre* ***Ruchi mam, Murli, Santosh(Sarkar) Subramanium , Sumant and Dr. Kuldeep,*** *for all lively and supportive interactions. Thanks to* ***Dr. Ansaullah,*** *Department of zoolgy,MSU for his helping hand during my crucial experiments.*

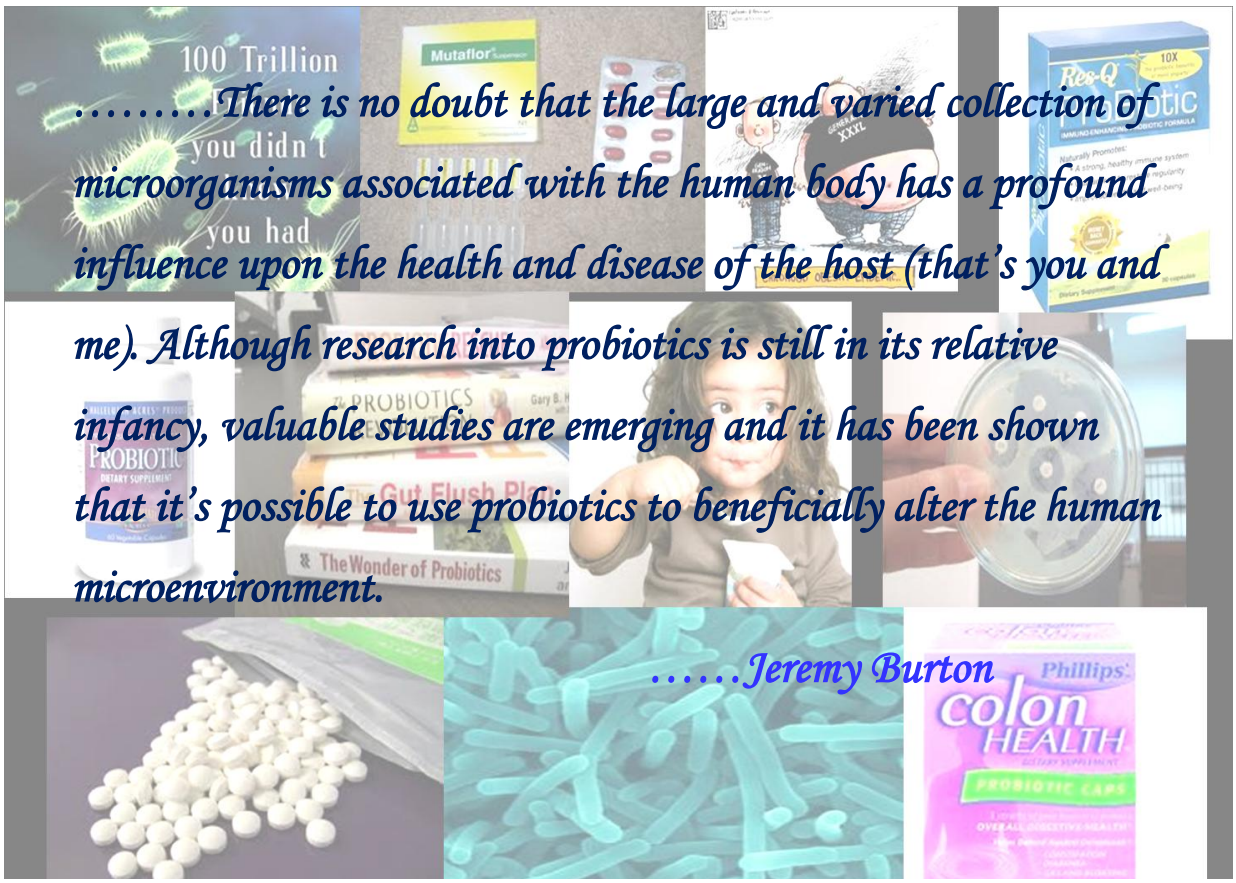
*I owe my research work to my dear **Parents** for their unconditional love and support, constant encouragement, difficult adjustments and for instilling in me, the moral values, confidence, faith, sense of responsibility, without which this work would not have been possible.*

***Priyanka**, my better half, at home played a major role in constantly supporting and refreshing the exhausted mind at the end of hectic daily work schedule.*

Thanks are due to my near and dear ones and other family members for having faith in my abilities.

Ultimately, I bow down to God! The Almighty, who bestowed me with a healthy mind and body as well as the strength to stand up to the expectations of my parents, teachers, family and friends.

Prasant Kumar



.....There is no doubt that the large and varied collection of microorganisms associated with the human body has a profound influence upon the health and disease of the host (that's you and me). Although research into probiotics is still in its relative infancy, valuable studies are emerging and it has been shown that it's possible to use probiotics to beneficially alter the human microenvironment.

.....Jeremy Burton

Metabolic Engineering- The field which was once considered to be "Information- limited" is now said to be "Imagination-limited".

List of Abbreviations used:

Amp	Ampicillin
APS	Ammonium persulfate
ARC	Aerobic Respiratory Control
ATP	Adenosine triphosphate
BLAST	Basic local alignment search tool
bp	base pair (s)
CCl_4	Carbon tetra chloride
CTAB	Cetyl trimethylammonium bromide
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
DTT	1, 4-Dithiothreitol
EcN	*E. coli* Nissle 1917
ED	Entner-Doudoroff
EDTA	Ethylene diamine tetra acetic acid
FNR	Fumarate Nitrate Reductase
FOS	Fructose Oligosaccharide
GFP	Green fluorescent protein
GI	Gastrointestinal Tract
GM	Genetically Manipulated
HDL	High Density Lipoprotein
IFN	Interferon ϒ
IL	Interleukin
inuJ	Inulosucrase
IPTG	Isopropyl β-D thio galactopyranoside
kb	kilobase (s)
kDa	Kilodalton
LAB	Lactic acid Bacteria
LB	Luria broth
LDL	Low Density Lipoprotein
LPS	Lipopolysaccharide
MDL	Malondialdehyde
mM/M	Millimolar/Molar
ng	Nanogram
nm	Nanometer
OD	Optical Density
OGTT	Oral Glucose Tolerance Test
Ori	Origin of replication
PAGE	Polyacrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
PCR	Polymerase Chain Reaction
RE	Restriction Endonuclease
rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate

SGOT	Serum Glutamic Oxaloacetic Transaminase
SGPT	Serum Glutamic Pyruvate Transaminase
Str	Streptomycin
TG	Tiglyceride
Tris	Tris (hydroxymethyl) aminomethane buffer
VHb	*Vitreoscilla* haemoglobin
VLDL	Very Low Density Lipoprotein
WHO	World Health Organization
WT	Wild type
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D galactopyranoside
µl/ml	Microlitres/millilitres
µg	Microgram

Note: The full forms of several rarely used abbreviations have been described within the text.

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CHAPTER 1

Review of Literature

1 Introduction

1.1 Probiotics

The term probiotic is derived from the Greek and literally translates as *for-life*. The probiotic was conceptualized at the end of 19th century by Elie Mechnikoff at the Pasture Institute, Paris. Metchnikoff is regarded as the grand father of modern probiotics. The scientific rationale for the health benefit of lactic acid bacteria were provided in his book. "The Prolongation of Life" published in 1907. He suggested, "The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the microbiota in our bodies and to replace the harmful microbes by useful and beneficial microbes". Contemporary of Metchnikoff, Henry Tissier observed that children with diarrhoea had in their stools a low number of bacteria characterized by atypical, Y shaped morphology. On the contrary, these "bifid" bacteria were abundant in healthy children. He suggested that these bacteria could be administered to patients with diarrhoea to help restore a healthy gut flora. In 1965, term 'probiotics' was first used by Lilly and Stillwell in a different context to represent the 'Substances secreted by one organism which stimulate the growth of another'. Nine years later, Parker described probiotics as "organisms and substances that contribute to intestinal microbial balance" (Anukam and Reid, 2007). Fifteen years later, Fuller proposed that probiotics were 'Live microbial supplements which beneficially affects the host animal by improving its microbial balance. In 2002, FAO/WHO defined Probiotics as 'live micro-organisms which when administered in adequate numbers confer a health benefit on the host'.

1.2 Current global and national status of Probiotics

The most common form for probiotics are dairy products mainly fortified foods. However, tablets, capsules, powders and sachets containing the bacteria in lyophilized form are available worldwide (**Table 1.1**) (Sander, 2005; Sekhon *et al*., 2010; Bhadoria *et al*., 2011). Worldwide market of probiotics is growing fast, however, in India; the market has just started to conceive with leading companies like Yakult, Amul, Nestle, and Mother dairy making the first step (Sekhon *et al*., 2010; Bhadoria *et al*., 2011; Raja *et al*.,

2011). In India, these companies have come up with their probiotic products, which are very popular. Some of the products are mentioned (**Table 1.2**).

Table 1.1: Probiotic products marketed worldwide with targeted health benefits (Sanders, 2007; Sekhon *et al*., 2010)

Strain	Brand Name	Producer	Proven effect in humans
Bifidobacterium animalis DN 173 010	Activia	Danone	Stabilises intestinal passage
Bifidobacterium breve Yakult	Bifiene	Yakult	
Bifidobacterium infantis 35624	Align	Procter & Gamble	Irritable Bowel Syndrome (IBS)
Bifidobacterium lactis HN019 (DR10)	Howaru™ Bifido	Danisco	Immune stimulation
E. coli Nissle 1917	Mutaflor	Ardeypharm	Immune stimulation, IBS, Ulcerative colitis.
Lactobacillus casei F19	Cultura	Arla Foods	Improves digestive health, immune stimulation, reduces antibiotic-associated diarrhoea, induces satiety, metabolizes body fat, reduces weight gain
Lactobacillus casei Shirota	Yakult	Yakult	Immune stimulation
Lactobacillus plantarum 299V	GoodBelly ProViva	NextFoods Probi	IBS, used post-operative
Lactobacillus rhamnosus ATCC 53013 (discovered by Gorbach & Goldin (=LGG))	Vifit and others	Valio	Immune stimulation, alleviates atopic eczema, prevents diarrhoea in children and many other types of diarrhoea
Lactobacillus rhamnosus LB21	Verum	Norrmejerier	Immune stimulation, improves digestive health, reduces antibiotic-associated diarrhoea

Veillonella parvula, Streptococcus and *Lactobacillus*	VSL#3	(VSL Pharmaceutical Inc., Fort Lauderdale, FL)	Effective for the management of remission of pouchitis and colitis

Table 1.2: Probiotic products marketed in India (Sekhon *et al*., 2010; Bhadoria *et al*., 2011; Raja *et al*., 2011)

Sl. No	Probiotic Products	Company
1	Probiotic drugs	Heritage Foods (India) Ltd
2	'b-activ' Probiotic curd	Mother dairy
3	'Nesvista' Probiotic Yougurt	Nestle
4	Probiotic ice creams, 'Amul Prolife' 'Prolite' and 'Amul Sugarfree'	Amul (Brand of Gujarat cooperative milk marketing federation Ltd)
5	Yakult Probiotic curd with *L. casei* strain *Shirota*	Yakult Danone India (YDL) Private Ltd
6	Probiotic drugs	Ranbaxy (Binifit)
7	Probiotic drugs	Dr. Reddy's Laborotries
8	Probiotic drugs	Zydus Cadila
9	Probiotic drugs	UniChem
10	Probiotic drugs	JB Chem
11	Probiotic drugs	GalaxoSmithKline
12	Fructo-Oligo Saccharides, Probiotic drugs	Genmark Alkem Labs

Major factors driving the growth of the global probiotics market are increasing levels of health-consciousness, and the availability of probiotics in the form of dietary supplements. Global industry analysts reported that in 2008, the global probiotics market (including both foodstuffs and supplements) was worth over US$15.7bn, or over 18% of the global functional foods (Starling, 2010). Since 2003, the global probiotics market has more than doubled in value terms, and is currently rising by almost 15% per annum and expecting to reach approximately worth of US$30 bn by 2015.

1.3 Characteristics of Probiotics

Probiotic organisms require certain characteristics to enable them to exert maximum therapeutic effects. These qualities are outlined in **Table 1.3**.

Table 1.3: Desirable properties of probiotics (Ouwehand *et al*., 2002)

Property	**Benefit**
Resistance to pancreatic enzymes, acid and bile	Survival of passage through the intestinal tract
Adhesion to the intestinal mucosa	Immune modulation Pathogen exclusion Enhanced healing of damaged mucosa Prolonged transient colonization (?)
Human origin	Species specific interactions with the host
Documented health effects	Proposed health effects are ‘true’
Safe	No health risk to consumer
Good technological properties	Strain stability Production at large scale Oxygen tolerance

1.4 Groups of Probiotics

The major groups of probiotics are *Lactobacilli*, *Bifidobacteria* and minor group is represented by *Saccharomyces, Streptomyces, E. coli*, although it is important to remember that there are many other types of bacteria that are also classified as probiotics. Each group of bacteria has different species and each species has different strains.

1.4.1 Lactic acid bacteria (LAB)

Lactic acid bacteria are characterized as Gram-positive, aerobic to facultative anaerobic, asporogenous rods and cocci which are oxidase, catalase, and benzidine negative, lack cytochromes, do not reduce nitrates to nitrite, gelatinase negative, and are unable to utilize lactate. They are used in the manufacture of dairy products such as acidophilus milk, yogurt, buttermilk, and cheeses. They are sub-divided into four genera *Streptococcus*, *Leuconostoc*, *Pediococcus* and *Lactobacillus*. Beneficial effects of lactic acid bacteria include alleviation of lactose intolerance (Batista *et al*., 2008; Granato *et al*., 2010; Pathan *et al*., 2011), prevention and treatment of diarrhea (Guarino *et al*., 2009; Pathan *et al*., 2011), maintenance of normal intestinal flora (Khurana *et al*., 2007), antagonism against pathogens (Zoumpopoulou *et al*., 2008), stimulation of the immune system (Dı´az-Ropero *et al*., 2006), anticarcinogenic activity (Commane *et al*., 2005), and reduction of serum cholesterol levels (Nguyen *et al*., 2007; Pan *et al*., 2010; Parnami *et al*., 2011).

1.4.2 Bifidobacteria

Bifidobacteria are characterised as gram-positive, obligate anaerobes, non spore-forming, nonmotile, distinct Y-shaped sometimes in the form of club-shaped. They optimally grow in anaerobic conditions while some species can only tolerate O_2 only in the presence of CO_2. The optimum growth temperatures are 37-41°C; minimum growth temperatures 25-28°C and a maximum at 43-45°C. Optimum pH for initial growth is 6.5-7.0 with no growth at pH 4.5-5.0 or 8.0-8.5. Genus *Bifidobacteria* contains approximately 30 species (Leke *et al*., 2007). A number of health benefits have been claimed for *Bifidobacterium* spp.. and therefore inclusion of these organisms in the diet is considered to be important in maintaining good health. *Bifidobacteriun* spp.. has anticarcinogenic properties, a specific probiotic effect, which are of three types: (1) elimination of procarcinogens; (2) modulation of procarcinogenic enzymes; and (3) tumour suppression (Wollowski *et al*., 2001; Shah, 2007). Furthermore, consumption of these organisms is an ideal method to re-establish the balance in the intestinal flora after antibiotic treatment (Quigley *et al*., 2006). Many health promoting affects have been attributed to certain

Bifidobacterium spp.. These include stimulation of the immune system, alleviation of lactose intolerance and prevention of gastrointestinal (GI) disorders (Shah, 2007).

1.4.3 Others

1.4.3.1 *Saccharomyces boulardii*

Saccharomyces boulardii is a probiotic, non-colonizing yeast species closely related to Brewer's yeast but not related to *Candida*. It aggressively displaces problematic yeast species in the GI tract and has been used to prevent acute diarrhea (Billoo *et al*., 2006; Czerucka *et al*., 2007). Oral consumption of *S. boulardii* results in its establishment in GI tract and secretes lactic acid and some B vitamins. It is eliminated after supplementation is discontinued. During use, friendly probiotic bacteria are allowed to colonize in the GI tract, supporting micro-ecology. *S. boulardii* increases the levels of secretory IgA and is effective against specific unfriendly microbes (Czerucka *et al*., 2007).

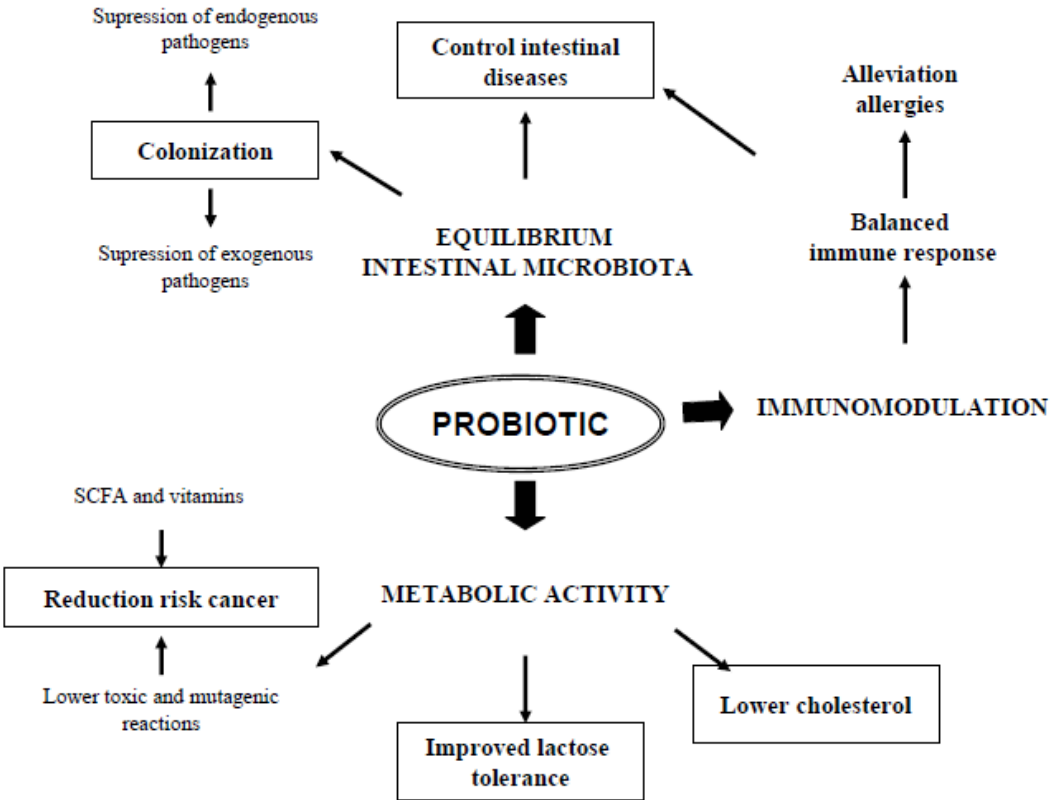
1.4.3.2 *Streptococcus gordonii*

Streptococcus gordonii belong to the viridian group of *Strepotococci*, nonpathogenic and commensal, which are integral members of the human oral flora. These organisms colonize tooth surfaces by creating biofilms in the human mouth, also known as dental plaque. *S. gordonii* plays an integral role in initiating colonization by creating surfaces for other colonizers to adhere to. The efficiency of *S. gordonii* in colonizing the oral cavity creates great potential for the stimulation of the mucosal immune system (Magliani *et al*., 1997). *S. gordonii* is a good candidate for a "live bacterial mucosal vaccine vector". *S. gordonii* can also be easily controlled genetically to produce a "fusion construct" that will enhance its stability. Another advantage is that the activation can be attributed to multiple immunostimulatory components present within *S. gordonii* bacterial cells thereby expressing a number of viral and bacterial antigens.

1.5 Mechanism of action of Probiotics

The mechanism by which Probiotics exert its effects are schematically represented in **Fig 1.1**: (i) The indigenous GI tract microflora stimulate the immune system of the host to respond more rapidly against potential pathogens (Collado *et al*., 2009); (ii) Probiotics can inhibit the growth of their competitors by producing antimicrobial substances called bacteriocins (Collado *et al*., 2009); (iii) Compete for adhesion to the intestinal brush border epithelium surface. Adherent non-pathogenic bacteria (probiotics) can prevent attachment and subsequent entry of pathogenic enteroinvasive bacteria into the epithelial cells (Guarner and Malagelada, 2003) and (iv) Bacteria compete for nutrient availability in ecological niches (Fooks and Gibson, 2002). Increasing the numbers of friendly bacteria by way of a probiotic may thereby decrease the substrate availability for other bacterial populations, especially pathogenic ones.

Fig. 1.1: Mechanism by which probiotics safeguard our health (Collado *et al*., 2009)



1. 6 Application of Probiotic

Clinical studies of Probiotic proved their potential application in GI disorders, including diarrhea, IBS, urogenital infection, *Helicobacter pylori* infection, stimulate the immune system, reduction in serum cholesterol and lactose intolerance **Table 1.4.**

Table 1.4: Selection of probiotic strains fulfiling the FAO/WHO Guidelines (Sanders *et al*., 2005; Carmen *et al*., 2010)

Strains	**An examples of Clinical evidence showing Probiotic effects**
Lactobacillus casei Shirota (Yakult, Japan)	Improvement in treatment of constipation
L. casei DN114 001 (Danone, France)	Reduced duration of winter infections in elderly subject
Lactobacillus rhamnosus GG (Valio, Finland)	Various benefits including improved treatment of diarrhoea and management of atopy
L. rhamnosus HN001 (Danisco, Denmark)	Enhanced immunity in the elderly, as measured by in vitro phagocytic capacity of peripheral blood polymorphonuclear leukocytes and tumoricidal activity of natural killer cells, following 3 weeks intake of probiotic
FL. rhamnosus 19070-2 and *Lactobacillus reuteri* DSM 12246 (Chr. Hansen, Denmark)	Reduction in acute diarrhoea in children following twice daily treatment
L. rhamnosus GR-1 and *L. reuteri* RC-14 (Chr. Hansen, Denmark)	Reduction in, and better treatment of, urogenital infections in women taking oral lactobacilli daily for 2 months
L. salivarius spp.. *salivarius* CECT5713	Recovery of inflamed tissue in TNBS model of rat colitis, increase in TNF-α and iNOS (inducible NO synthase) expression
L. reuteri ATCC 55730 (BioGaia, Sweden)	For treatment of diarrhoea and to produce CD4-positive T- lymphocytes in the ileal epithelium

Lactobacillus plantarum 299V (Probi, Sweden)	Decreased incidence of infections in liver transplant patients
Lactobacillus acidophilus La5 (Chr. Hansen, Denmark)	Suppression of *Helicobacter pylori* with use of La5 and B. lactis BB12 yogurt given twice daily after a meal for 6 wks
L. acidophilus L1 (Campina Melkunie, Holland)	Fermented milk containing this organism was found to lower serum cholesterol – this would translate to 6–10% reduction in risk for coronary heart disease
Lactobacillus johnsonii La1 (Nestle, Switzerland)	A moderate but significant difference in *H. pylori* colonization was detected in children receiving live La1
Lactobacillus paracasei LP-33 (Uni-President Enterprise Corp., Tainan, Taiwan)	Effectively and safely improved the quality of life of patients with allergic rhinitis
Lactobacillus brevis CD2 (VSL Pharmaceuticals, Inc., Fort Lauderdale, FL).	Decreases *Helicobacter pylori* colonization, thus reducing polyamine biosynthesis
Lactobacillus gasseri OLL 2716 (Meiji Milk Products, Tokyo, Japan).	Yogurt containing this organism suppressed *H. pylori* and reduced gastric mucosal inflammation
L. fermentum, L.reuteri	Improvement of histology in a TNBS model of rat colitis, decreased levels of TNF-α and i-NOS expression
VSL#3 (VSL Pharmaceuticals, Inc., Fort Lauderdale, FL)	Effective for the management of remission of pouchitis and colitis
Saccharomyces cerevisiae boulardii lyo (Biocodex, France)	250 mg treatment for 5 days reduced the duration of acute diarrhoea and the duration of hospital

Bifobacterium animalis/lactis BB12 (Chr. Hansen, Denmark/Nestle, Switzerland)	Various effects including prevention and treatment of diarrhoea
B. animalis/lactis DN-173 010 (Danone, France)	Two to three servings per day helps with regularity
Bifidobacterium longum BL1 (Morinaga, Japan)	3 x 100 mL per day low-fat drinking yogurt prepared with the two starter cultures plus *B. longum* BL1 resulted in some evidence of lower serum cholesterol
Bifidobacterium lactis HN01	The ex vivo phagocytic capacity of mononuclear and polymorphonuclear phagocytes and the tumoricidal activity of natural killer cells were elevated
Bifobacterium infantis 35624 (Ardeypharm, Germany)	Taken in a malted milk drink for 8 weeks, shown to relieve abdominal pain/discomfort,bloating/distention, and bowel movement.
BIFICO (3 bifidobacteria species)	Prevention of flare-ups of chronic ulcerative colitis, inactivation of NF-κB, decreased expressions of TNF-α and IL-1β and elevated expression of IL-10
L. rhamnosus GG, L. rhamnosus Lc705, P.freudenreichii spp.. *shermanii JS and B. breveBb99*	Alleviating irritable bowel syndrome symptoms
E. coli Nissle 1917	use in treatment of colitis

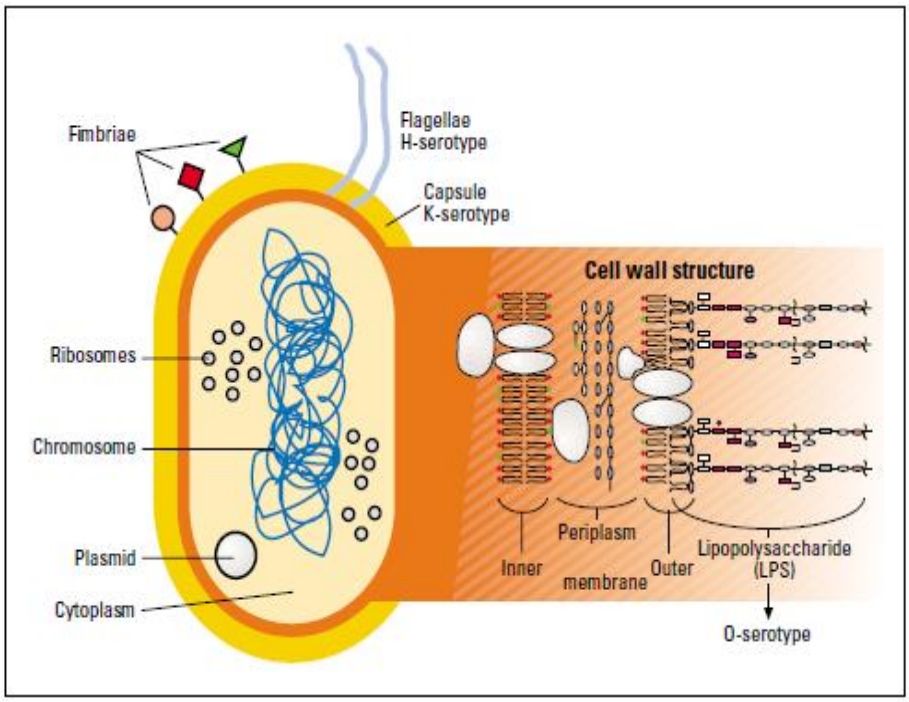
1.7 *Escherichia coli*

Theodor Escherich delivered a lecture to the Society of Morphology and Physiology in Munich bearing the title “The intestinal bacteria of the neonate and infant”

in 1885. He reported on his research results meconium of the neonate is sterile, but is colonised by microorganisms in the first few hours of life. He noticed mainly two types of bacteria in the infant faeces, which he described as *"obligate species of the faeces of milk-fed infants"*. One of these was found particularly frequently in the lower sections of the gut – as he described them as typical "colonic bacteria" and gave them the species name *"Bacterium coli commune"*, the common colonic bacterium later rename as *Escherichia coli* in 1919. On the basis of his findings, Escherich described the colonisation of the upper sections of the intestine as monotonous and sparse, and stated that the rapid increase in bacteria begins at the ileocaecal valve. He postulated that bacterial multiplication was not dependent on the supply of food and that the bacteria would meet their nutritional requirements from the metabolisation of intestinal secretions.

The *E. coli* bacterium is a gram-negative rod of about 1.1–1.5 μm x 2.0 – 6.0 μm in size. It grows under aerobic and anaerobic conditions (facultatively anaerobic), because it possesses two different redox systems (menaquinone and ubiquinone) which enable it to derive energy from catabolic metabolism under both aerobic and anaerobic conditions. Under optimal growing conditions, the rate of cell division of the *E. coli* bacteria is very fast: the number of bacterial cells can double every 20 minutes. However, the circumstances that are ideal for this population dynamics are not achieved in the bacteria's normal environment. Midtvedt, in 1998 reported that doubling of cells in the caecum of the rat after about 100 minutes, while in the human gut it may take 30 hours. Various strains of *E. coli* have been classified serologically on the basis of their surface antigens O, K and H. O antigens represent the heat-stable constituents of the lipopolysaccharide complex (LPS) of the outer cell membrane, K antigens represent polysaccharides of the capsule and H antigens represent whip or flagellar antigens (**Fig. 1.2**).

Fig. 1.2: Diagramatic longitudinal cross-section and cell wall structure of a gram-negative *E. coli* serotype O6:K5:H1 (Schulze *et al*., 2006).



According to Srivastava *et al*., (2009) around 50,000- 100,000 different serotypes may occur in nature arising from the combination of different antigen structures. To date 173 surfaces (O), 103 capsular (K) and 56 flagellar (H) antigens are known in *E. coli*. In addition, there are also more than 100 adhesin variants cause further differences in serological behaviour and exhibit differences in receptor recognition. However, the number of pathogenic serotypes is limited.

Escherich was convinced that *E. coli* is a "harmless commensal" (Tenaillon *et al*., 2010). He was right because most of the approximately 50,000 serotypes mentioned must be counted among the commensal gut organisms. However, Escherich reported *"on cystitis in children provoked by E. coli*. He hypothesised that the intestinal bacteria could be considered as a source of urinary tract infections (bladder and kidney infections).

Pathogenic *E. coli* variants are characterised by the presence of various virulence factors, such as various toxins, particular fimbrial adhesins, invasins or secretion systems. *E. coli* pathogenic in the intestine are divided at present, on the basis of their virulence factors discovered in recent years, into 6 classes, which cause diarrhoeal illnesses with different clinical manifestations (Kaper *et al*., 2004). Pathogenic *E. coli* present in the small intestine are divided into six classes' based on their virulence factors, which cause diarrhoeal illnesses.

1.7.1 Intra- and extra-intestinal pathogenic *E. coli* variants (Kaper *et al*., 2004).

Enterotoxic *E. coli* (ETEC):

These *E. coli* cause diarrhoea in infants and travelers (travelers' diarrhoea) in underdeveloped countries or regions of poor sanitation. ETEC infections manifest themselves as minor discomfort to a severe cholera-like syndrome. Enterotoxins produced by ETEC include the LT (heat-labile) toxin and/or the ST (heat-stable) toxin which cause damage to enterocytes.

Enteraggregative *E. coli* (EAggEC):

These *E. coli* cause persistent watery diarrhoea in young children. EAggEC adhere to the intestinal mucosa and cause non-bloody diarrhoea without invading or causing inflammation. The organism produces a toxin, which resembles the heat stable toxin of ETEC.

Enteroinvasive *E. coli* (EIEC):

Closely resemble *Shigella* in their pathogenic mechanisms and the kind of clinical illness they produce. They penetrate and multiply within epithelial cells of the colon causing widespread cell destruction. The clinical syndrome is identical to *Shigella* dysentery and includes dysentery-like diarrhoea with fever. Like *Shigella*, EIEC are invasive organisms, but they do not produce LT or ST toxin and, unlike *Shigella*, they do not produce the shiga toxin.

Enteropathogenic *E. coli* (EPEC):

Induce watery diarrhoea similar to ETEC, but they do not possess the same colonization factors and do not produce ST or LT toxins. They make use of specific

fimbrial adhesins called bundle-forming pili (*bfp*) to dock onto the intestinal mucosa and inject signal proteins into the epithelial cell that leads to destruction of the cell.

Enteroheamorrhagic *E. coli* (EHEC):

These are the causative agent of bloody diarrhoea. They enter the mucosal cell and release toxins similar to the shiga toxin and enterohemolysin, leading to bloody lesions in the intestine.

Diffusely Adherent *E. coli* (DAEC):

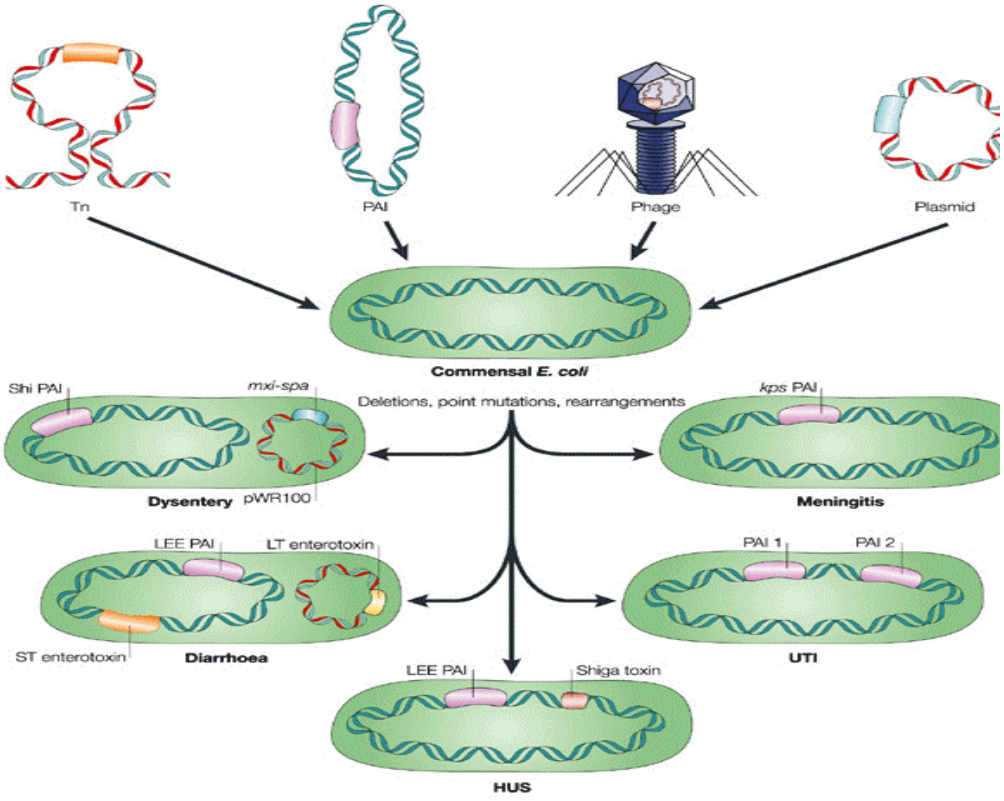
E. coli (DAEC) strains are a major cause of urinary tract infections worldwide, but its role as a causative agent of diarrhoea is controversial.

1.7.2 *E. coli*: Transition between commensalism and pathogenicity

Host and the environment determine the genetic structure of commensal *E. coli* (Tenaillon *et al*., 2010). After sequencing the complete genome of the *E. coli* K-12 strain MG 1655 in 1997, genomes of non-pathogenic and pathogenic variants of the species were decoded. Analysis of the genome sequence data led to the realisation that bacterial genomes frequently consist of a core genome, but possess DNA sequences that have possibly been integrated into the genome by horizontal gene transfer. Mobile genetic elements are frequently involved in horizontal gene transfer of virulence-associated genes. Acquisition of genetic information as a result of lateral spread of mobile genetic elements contributes to constant and rapid evolution of bacterial pathogens. The integration of foreign DNA by means of mobile genetic elements (e.g.plasmids, bacteriophages, transposons), mutations and intra-genomic relocations could make a significant contribution to the creation of a flexible gene pool (Kaper *et al*., 2004). When pathogenic variants come into being by horizontal gene transfer what are known as the "pathogenicity islands" (PAIs - large chromosomal DNA regions with DNA sequences which differ greatly in their composition from the core genome) also play a crucial role **Fig 1.3**. Large clusters of virulence PAIs can be found on plasmids or integrated into the chromosome in pathogenic bacteria but they are not found in

commensal bacteria (Hacker and Kaper, 2000). PAIs are usually flanked by mobile genetic elements such as bacteriophages, insertion sequences or transposons and often insert near tRNA genes.

Fig.1.3: *E. coli* virulence factors encoded by mobile genetic elements (Tn): Heat stable enterotoxin (ST) of ETEC), plasmids (for example, heat-labile enterotoxin (LT) of ETEC and invasion factors of EIEC), bacteriophage (for example, Shiga toxin of EHEC) and pathogenicity islands (PAIs) — for example, the locus of enterocyte effacement (LEE) of EPEC/EHEC and PAIs I and II of UPEC. Commensal *E. coli* can also undergo deletions resulting in 'black holes', point mutations or other DNA rearrangements that can contribute to virulence. These additions, deletions and other genetic changes can give rise to pathogenic *E. coli* forms capable of causing diarrhoea (EPEC, EHEC, EAEC, and DAEC), dysentery (EIEC), haemolytic uremic syndrome (EHEC), urinary tract infections (UPEC) and meningitis (MNEC). HUS, haemolytic uremic syndrome; UTI, urinary tract infection (Kaper *et al*., 2004).



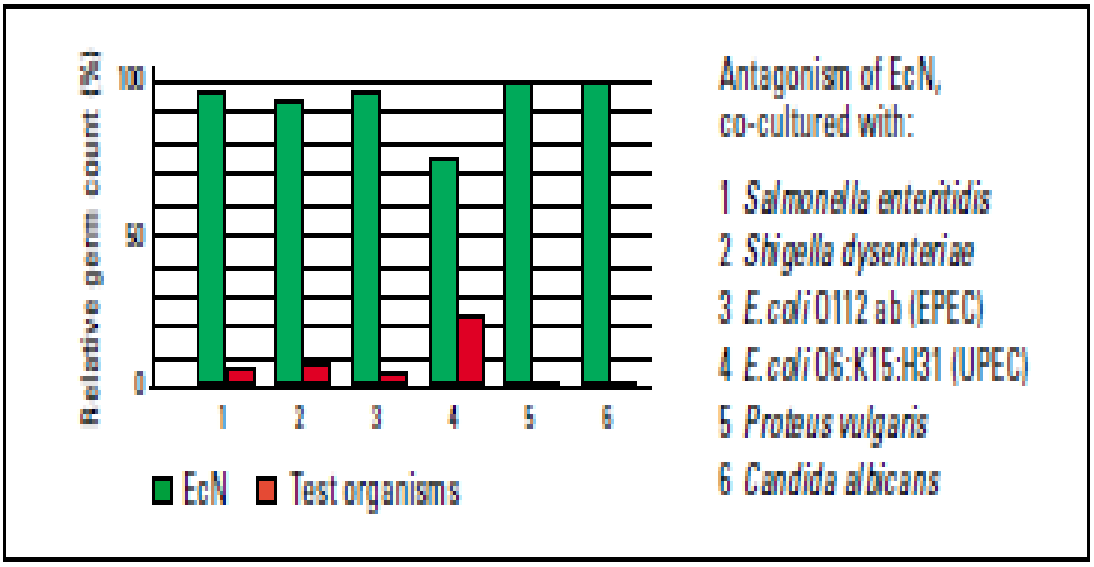
1.8 *Escherichia coli* as probiotic and its clinical significance

1.8.1 *Escherichia. coli* Nissle 1917

During the end of the 19th century Medical Microbiology experienced a tremendous upswing, mainly due to the pioneering discoveries of Pasteur, Koch, Escherich, Ehrlich, Metchnikoff and many others. However the question remained unanswered whether the intestinal flora as a whole or certain intestinal micro-organisms tend to have a positive influence, or more of a negative influence, on the host, and equally unanswered was the question concerning the mechanisms regulating the bacteria that co-exist in the GI tract (Sonnenborn and Schulze, 2009). Alfred Nissle, a bacteriologist, raised further questions on the basis of his studies of intestinal micro-organisms. "Why and how do bacteria mutually influence each other in their growth in a mixed culture such as is present in the intestine?" or "Why do the intestines of just a few people remain healthy in the face of epidemic-like diarrhoeal diseases?" After working experimentally on these questions for several years, Nissle presented his lecture entitled "On the principles of a new causal control of pathological intestinal flora" Nissle had developed an "Antagonism Index" to identify these intestinal micro-organisms and found "more strongly" and "more weakly" antagonistic strains among the *E. coli* strains he had isolated from the intestinal tract. He also used this method to determine the antagonistic activity of various *E. coli* bacteria against each other. During the First World War he found *E. coli* strains with a particularly strong inhibitory action in two patients who had *"never shown any inclination to intestinal diseases and had specifically not contracted any infectious intestinal process when a fairly large proportion of those around them had contracted such infections, and who were exposed to infection to a most substantial degree as a consequence of living in close contact with those already sick"*. Nissle tested these strains, which also retained their antagonistic effectiveness under laboratory conditions, firstly on himself and on healthy individuals in order to establish their safety to humans following oral administration. Subsequently this, he treated patients experimentally, firstly those with infectious diarrhoea, paratyphoid fever B, shigellosis and chronic salmonella carriers, and later patients with post-dysenteric intestinal

dysfunction ("irritable colon") or chronic habitual constipation. Nissle obtained another *E. coli* isolate with a particularly strong "antagonistic potency" from the faeces. He thereby created the basis for a medicine containing live *E. coli* bacteria as its active principle. Nissle registered the name "MUTAFLOR" for this medicine, and it acquired protection as a trademark on 1st March 1917. Later the strain of *E. coli* contained in Mutaflor® was given the strain designation "Nissle 1917", abbreviated to "EcN", acted *in vitro* against various pathogenic *E. coli* strains (**Fig 1.4**).

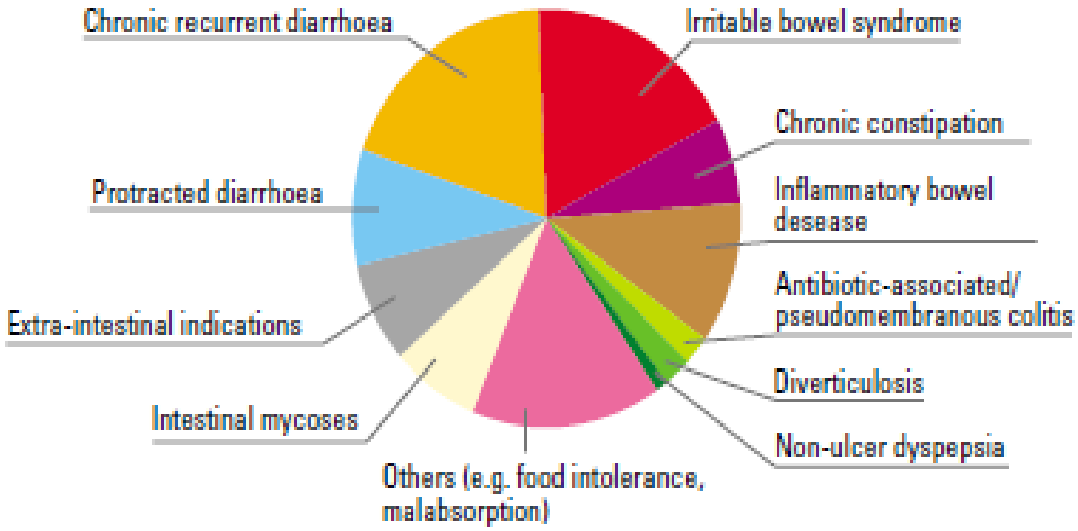
Fig.1.4: Antagonism of *E. coli* Nissle 1917 (EcN) against various micro-organisms under *in vitro* condition (Schulze *et al*., 2006)



Determination of non-pathogenicity or non-virulence is of particular importance in therapeutic use of *E. coli* as probiotic. Advances in molecular genetics differentiation and identification methods enabled to distinguish pathogenic *E. coli* variants unequivocally from non-pathogenic strains. In addition, non-pathogenic strains such as *E. coli* Nissle 1917 exhibit no harmful effects in toxicological studies of both conventionally kept and germ-free animals (Schulze *et al*., 2006). *E. coli* Nissle 1917 (EcN), fulfils all characteristics of probiotic mentioned in **Table 1.3**. Genome sequence revealed the

absence of pathogenicity factors (e.g. of enterotoxins, haemolysins, cytotoxins, invasins, pathogen-specific fimbriae), combined with the presence of "fitness factors" (e.g. microcins, iron uptake systems, typical adhesins) which enable the micro-organism to survive in and colonise the intestine (Grozdznov *et al*., 2004). *E. coli* strain Nissle 1917 has been used widely in human as a therapy for dirrahoea, inflammatory bowel disease, constipation and ulcerative colitis (**Fig 1.5**).

Fig 1.5: Therapeutic potential of Mutaflor® (Schulze *et al*., 2006)



1.8.2 *Escherichia coli* strain M-17 (EC-M17) is a novel probiotic drug with beneficial effects on the GI tract. EC-M17 is believed to be a direct descendant of the M17 strain first identified by the Russian bacteriologist L. G. Peretz in 1933 (Fitzpatrick *et al*., 2008). This strain used extensively in humans as a therapy for GI diseases such as colitis, inflammatory bowel disease and infections. Anti-colitis action of EC-M17 is mediated by modulation of immune processes attributed to an inhibitory effect on NF-kB signalling.

1.8.3 *Escherichia coli* H22 inhibits pathogenic or potentially pathogenic strains of at least seven genera of the family Enterobacteriaceae (*Enterobacter*, *Escherichia*, *Klebsiella*, *Morganella*, *Salmonella*, *Shigella*, and *Yersinia*) in both *in vitro* and *in vivo*

conditions. Although the nature of *in vivo* inhibition remains unclear, the *in vitro* inhibition of these strains has been shown to be mediated by production of microcin C7 (Smajs *et al*., 2008), colicins E1 and Ib, aerobin and an unidentified phage (Cursino *et al*., 2006). Simultaneous administration of the probiotic and the enteric pathogen *Shigella flexneri* to germ-free mice resulted in a strong inhibition of the pathogen, which was attributed to its microcin production. Thus *E. coli* strain H22 acts as one of the most potent probiotic strain for livestock and humans.

1.9 Significance of *E. coli* in Genetic engineering

The importance of *E. coli* to international scientific advance is also underlined by the fact that 14 Nobel prizes have been awarded in the last 50 years for work on and with *E. coli*. Human insulin could be manufactured with many improvisations in the genetic modifications of *E. coli* (Miralles *et al*., 2009). Genetic engineering and biotechnology has now succeeded in manufacturing recombinant medicines (Garcia *et al*., 2009). Genetic engineering tools are well standardized for *E. coli* making it a preferred organism for metabolic engineering and developing computational programs or algorithms for the metabolic and regulatory networks (Baumler *et al*., 2011).

1.10 Genetic engineering of probiotics

Genetic engineered probiotic is of dual connotation: the genetic engineering of a previously non-probiotic to acquire probiotic properties and the genetic engineering of a known probiotic to enhance its probiotic properties, both leading to the creation of new, genetically manipulated (GM) probiotics. A number of studies have been reported that utilize a diverse combination of tools to design probiotics. Heterologous protein expression is an obvious approach (Paton *et al*., 2006). In this, one can make use of expression systems from native cDNA clones as well as of elaborate products of modern DNA technology such as synthetic genes and designed proteins. One can also expand the range of possible active components beyond protein therapeutics by engineering the metabolism of microorganisms through the integration of foreign enzymes. Metabolic engineering allows the use of, for example, modified lipopolysaccharide and anti-

inflammatory via the incorporation of new metabolic pathways (LeBlanc *et al*., 2010; Paton *et al*., 2011).

1.10.1 Sequestration of toxins

Shigella dysenteriae causes bacillary dysentery by Stx toxin which is composed of a catalytic A subunit, the actual toxin and a pentameric B subunit that is responsible for binding to its receptor, globotriaosyl ceramide (Gb3, or Galα [1→ 4]Galβ [1→ 4]Glc-ceramide, found in all human pathogens) or globotetraosyl ceramide depending on the Stx type. Antibiotic treatment leads to a sudden release of surface-associated Stx from the pathogen, which only aggravates the situation. By introducing the genes *lgt* C and *lgt* E from *Neisseria meningitidis* and *N. gonorrhoeae*, respectively, encoding glycosyl transferases that account for the incorporation of the oligosacharide structure Galα [1→4] Galβ [1→4]Glc into *E. coli*, a strain with an altered LPS structure was obtained (Paton *et al*., 2001). This strain displayed high binding activity for Stx. When administered twice daily to mice infected with STEC, the new strain provided complete protection.

1.10.2 Replacement therapy

Probiotic therapies can be grouped as 'replacement therapies', representing an approach whereby a noxious micro-organism is replaced in its ecological niche by a more potent but harmless competitor. Some of these therapeutic approaches are used in the treatment of various periodontal conditions like gingivitis and oral cavity disorders including tooth decay or periodontal disease (Fernandez *et al*., 2010). *In-vitro Lactobacillus rhamnosus GG* inhibits colonization by *Streptococcal* cariogenic pathogens, and therefore reduce tooth decay incidence in children (Fernandez *et al*., 2010). *L. reuteri*, effective in treatment of gingivitis and bacterial plaque deposition in patients with moderate-to-severe gingivitis.

1.10.3 Strategies that involve antibody production

Neutralizing antibodies that are directed towards a pathogen, toxin, cytokine or other agent have proved very valuable and specific tools in medicine (Steidler *et al*.,

2006).With the emergence of single chain (ScFv) antibody technology, it has now become possible to produce neutralizing antibodies from recombinant bacteria. Most of the work in this area relates to the production per se of the antibody for downstream processing and use as a purified protein. Owing to their structure, these peptides suffer from very short half lives *in vivo* hence suitable delivery systems are required to allow their use as therapeutics. A number of applications are now emerging in which the expressor strain itself is used for *in situ* production of the antibody fragment especially to control colonization by pathogens.

Candida albicans is a most frequent causative agent of mucosal inflamation in humans. Infections are seen in the mouth and oesophagus of immune compromised persons such as HIV infected subjects. *C. albicans* also causes acute vaginitis in otherwise healthy women. *S. gordonii* species with good vaginal colonization and heterologous expression potential *in vivo* used for the eradication of *C. albicans* infections (Beninati *et al*., 2000). Anti-idiotypic ScFv were produced, the surface of which resembled the structure of a wide-spectrum killer toxin of *Pichia anomala*. (Magliani *et al*., 1997). Two *S. gordonii* strains were constructed: one that expressed the ScFv at its surface and a second that secreted the ScFv. Both surface bound and secreted ScFv showed candidacidal activity over a wide concentration range. Both *S. gordonii* strains successfully colonized the vagina and cleared experimental *C. albicans* infection in rats, this being dependent on the presence of this ScFv.

1.10.4 Immune intervention

Immune intervention strategies are those in which the immune system is actively engaged for the acquisition of health benefits. Genetically modified *Lactococcus lactis* secreting interleukin 10 provides a therapeutic approach for inflammatory bowel disease. IL-10 is a strong anti-inflammatory cytokine that has shown promise in clinical trials for the treatment of IBD (LeBlanc *et al*., 2010). A probiotic strain of *E. coli*, engineered to secrete HIV-gp41-hemolysin A hybrid peptides, which block HIV fusion and entry into target cells. Administered orally or as a rectal suppository, this "live microbicide" (Lagenaur and Berger, 2005) colonizes the mucosa and secretes the peptide *in situ*,

thereby providing protection at the mucosal barrier, preventing the virus from entering the blood stream and causing a systemic infection. This approach can potentially provide protection in advance of HIV exposure for up to a month. Other anti-HIV probiotics currently in development include a genetically engineered *S. gordonii* which produces cyanovirin-N, a potent HIV-inactivating protein originally isolated from cyanobacterium, and a natural human vaginal isolate of *Lactobacillus jensenii* modified to secrete two-domain CD4 which inhibits HIV entry into target cells (Chang *et al.*, 2003).

CHAPTER 2

Isolation and Characterization of Potential Probiotic Escherichia coli Strains from Rat Faecal Samples

2.1 Introduction

The GI tract is a biologically diverse and complicated system which contains around 10^{14} bacterial cells and up to 1000 species (Backhand *et al*., 2005). The dominant microflora in faecal samples is obligatory anaerobic (*Bacteroides* spp., *Eubacterium* spp., *Bifidobacterium* spp., *Lactobacilli*) and anaerobic (*Cocci* and *Clostridium* spp.). In addition, facultative anaerobic organisms such as *E. coli*, *Enterococci* and *Streptococci* are also present (Kleessen *et al*., 2000). The microbial population consists of commensurate bacteria and opportunistic pathogens. Some of the commensurate bacteria with beneficial effects to the host have been employed as probiotics. Probiotic bacteria are friendly to the host and protect the host against infection by secreting antimicrobial substance (Klaenhammer *et al*., 1999). Antimicrobial substances include short chain fatty acid, hydrogen peroxide and bacteriocins. Bacteriocins have relatively narrow killing spectrum and are only toxic to bacteria closely related to the producing strain (Cascale *et al*., 2007). According to Klaenhammer, 99% of all bacteria may make at least one bacteriocin (Klaenhammer *et al*., 1999; Riley *et al*., 2002). Mode of action of bacteriocin produced from gram positive and gram negative microorganism differ and the immune responses activation in host against gram positive and gram negative also vary (Otte *et al*., 2004; Gillor *et al*., 2008). Consortium of organisms might be more effective than the application of a single strain because the protein released by gram positive and gram negative bacteria modulate the intestinal epithelial of the host by different mechanism including the competition of whole organisms for contact with the epithelial surface as well as stabilization of the cytoskeleton barrier function and the induction of mucin expression. The use of probiotic microbes to improve health is becoming well established. Most of the bacteria in commercial preparations are Gram-positive, however some enteric *E. coli* isolates, the normal GI microflora were also proved to be a useful as probiotic. A non-pathogenic *E. coli* strain Nissle 1917 has been well established in human medicine since 1917 (Buenau *et al*., 2005).

This strain was used successfully under the brand name Mutaflor for treating various gut-related diseases, e.g., chronic constipation, ulcerative colitis, Crohn's disease, or pouchitis. In addition, the strain prevented colonization of the intestine

with microbial pathogens in new born infants, reduce intestinal inflammation, strengthen the intestinal barrier against pathogen and boost up the host immune function (Buenau *et al*., 2005; Gronbach *et al*., 2010). In Germany, *E. coli* strain Nissle 1917 is authorized under the brand name Ponsocol for the prophylaxis of neonatal calf diarrhea and has been marketed since 2001. *E. coli* strain M-17 (EC-M17) is also a Probiotic agent with beneficial effects on the GI tract. EC-M17 is believed to be a direct descendant of the M17 strain first identified by the Russian bacteriologist L. G. Peretz in 1933 (Alder, 2006). This strain has been used extensively in humans as a therapy for GI diseases and infections. The Russian literature documents the use of EC-M17 in thousands of individuals, mostly for the treatment of dysentery.

E. coli H22 produces bacteriophage particles, aerobactin, colicins E1 and Ib, and microcin C7 type of antimicrobial compounds with inhibitory capabilities against pathogenic *Enterobacter* spp. and inhibited the GI enteric infections (Cursino *et al*., 2006). Bacteriocins inhibit pathogens within the closely related species such as *Salmonella*, *Shigella*, *Klebsiella*, *Enterobacter* and pathogenic *E. coli*, which are the most common cause of GI disorders.

This chapter deals with the isolation and characterization of potential probiotic of *E. coli* strains from rat faecal matter which could eliminate gram negative pathogens. For this purpose, *E. coli* strains were isolated and tested for probiotic properties such as antimicrobial activity against enteropathogens, antibiotic susceptibility and resistance to low pH, absence of virulence traits, susceptibility to proteolytic activity and detection of colicins type.

2.2 Materials and Methods

2.2.1 Isolation of *E. coli* from rats faecal samples:

Faecal samples were collected from Charles Foster rats of different age groups. Samples were dissolved in 10 mL of 0.85% NaCl and agitated vigorously. Serial dilutions of the resulting suspensions were spread on MacConkey lactose agar (HiMedia) and incubated for 24 h at 37°C. Approx 10-15 lactose-positive colonies from each sample were selected to perform IMViC tests. Isolates confirmed IMViC positives were finally transferred to HiCrome™ Coliform Agar (HiMedia). Isolated strains confirmed by above tests as *E. coli* were used to screen for their probiotic ability.

2.2.2 Testing for antimicrobial activity:

Detection of antimicrobial activity, agar spot tests were performed (Schillinger *et al*., 1989). Indicator strains were grown in Luria broth at 37°C and approximately $5x10^7$ cells were inoculated into 4 mL of soft agar containing 0.7% agar) and poured over the plate on which the producer was spotted. The plates were incubated at 37°C for 24 h. Inhibitory activity was demonstrated by a clear zone around the spots.

2.2.3 Testing for resistance to antibiotics:

E. coli strains were examined for resistance to amikacin (30 µg), amoxyclav (30 µg), ampicillin (10 µg), ceftazidime (30 µg), cephotaxime (30 µg), co-trimoxazole (25 µg),ciprofloxacin (5 µg), cefuroxime (30 µg), furazolidone (100 µg), gentamicin (10 µg), nitrofurantoin (300 µg), nalidixic acid (30 µg), netilin (30 µg), ofloxacin (10 µg) and tetracycline (30 µg) and tobramycin (10 µg) using commercial discs (HiMedia Laboratories). *E. coli* strain BL21 sensitive to all the antibiotics was included for quality control. Characterization of strains as susceptible, resistant or having reduced susceptibility was done in accordance with the manufacturer's instructions on sizes of inhibition zones around each disc, which matched the interpretive criteria recommended by the Clinical and Laboratory Standards Institute (CLSI).

2.2.4 Tolerance to acidic pH values:

E. coli strains were grown in Luria broth (HiMedia) at 37°C overnight, subcultured into fresh Luria broth and incubated till the culture was grown up to 0.6 OD at 550nm. The cultures were centrifuged at 5000 g for 10 min. The pellets were washed in sterile Phosphate-Buffered Saline (PBS) pH 7.0 and resuspended in PBS. Initial count was determined with appropriate dilution. Each strain was diluted 1/100 in PBS at pH 1.0, 2.0 and 3.0. Incubation times were 2, 4 and 6 h. Bacterial cultures were then transferred to Luria agar plates and incubated at 37°C overnight (Dunne *et al*., 2001; Mourad *et al*., 2006). The number of colonies was counted to obtain the viable counts at each time interval. Survival percentage of strains to different pH values was then calculated as, percentage survival = (viable counts after acid exposure/initial viable counts) x 100 (Schillinger *et al*., 1989; Dunne *et al*., 2001).

2.2.5. Detection of pathogenic strains:

PCR reactions were in a 25 µL volume and comprised 1 µL of $MgCl_2$ (15 mM), 0.5 µL of 2.5 mM dNTPs, 2.5 µL of *Taq* buffer (10X), 0.5 µL of each primer (25 pM each), 1 µL of *Taq* DNA polymerase (Bangalore Genei Pvt Ltd.), and 2 µL of the DNA sample. Polymerase chain reaction was done with appropriate temperature profile according to the melting temperature (Tm) of the primers as reported (Toma *et al*., 2003). Positive controls for 8 target genes were generated from DNA extracted from activated sludge and were identified on the basis of amplicon length (**Table 2.1**).

Table 2.1: Size of Amplicon target for identification of different *E. coli* pathogen

Primer	Amplicon (Size bp)	Target Gene	Strain
SK	881	*eae*	EPEC
VT	518	*stx*	STEC
AL	147	*est*	ETEC
LT	322	*elt*	ETEC
Ipa	619	*ipaH*	EIEC
Agg	254	*aggR*	EAEC
Eagg	194	*CVD432*	EAEC
Asp	282	*aspU*	EAEC

The PCR reaction mixture was incubated in a thermal cycler (Biorad) under the following cycling regime.

Lid temp – 105 ºC

Step -1 (Initial denaturation): - 96 °C - 3 min

1) Cycle: 1
- Step - 2 (Denaturation):- 94 °C – 1 min
- Step -3 (Annealing): - 52 °C - 1 min
- Step – 4 (Extension): - 72 °C - 1 min

Steps 2, 3, 4 were repeated for 30 cycles

Step -5 (Final extension): - 72 °C - 10 min.

2) For Enteropathogen (eae) 70 ^{0}C annealing temperature was used.

Cycle: 1
- Step - 2 (Denaturation):- 94 °C – 1 min
- Step -3 (Annealing): - 70 °C - 1 min
- Step – 4 (Extension): - 72 °C - 1 min

Steps 2, 3, 4 were repeated for 30 cycles

Step -5 (Final extension): - 72 °C - 10 min.

2.2.6. Characterization of the antimicrobial agent:

Samples of culture supernatants, pellets and crude extract were examined for susceptibility to proteolytic enzymes. The following enzymes were used: Proteinase K (5 mg mL^{-1}) and Trypsin (2 mg mL^{-1}) (Mourad *et al*., 2006). The crude extract was treated with enzymatic solutions and incubated at 37°C for 1h, after which the remaining activity was determined by the agar spot technique (Schillinger *et al*., 1989).

2.2.7. Detection and identification of Colicins:

Culture was grown overnight and freshly inoculated 50 µL in 5 mL in Minimal medium containing 0.6% casamino acid and 1% glucose and grown till 0.6 OD. This culture was split into two parts and one part was induced with Mitomycin C (200 ng mL-1). After 4 h of incubation at 37°C (for induced culture), the samples

were centrifuged at 15000 g/10 min/4°C. Supernatant was filtered with cellulose acetate filter (0.2 µm filter) and 5 µL was spotted on the indicator organism i.e., *Salmonella* sp, *Klebisella pneumoniae*, *Staphylococcus aureus* and *Enterobacter asburiae*. The pellet was suspended in 0.05 M Phosphate buffer pH 7.0 and sonicated 30-60 sec and centrifuged at 15000 g/10 min/4°C, lysis culture was filtered with cellulose acetate filter (0.2 µm filter) and 5 µL was spotted on the indicator organism. PCR based method was used for identification of 18 different colicins using 9 primers (Setia *et al*., 2009) (**Table 2.2**).

Table 2.2: Primers used for amplification of colicin genes

Target gene	Primer Name	Primer sequences (5' to 3)	PCR Product Size, bp	Annealing temperature, °C
Colicin A, N, S4	NS4f NS4r	CGTAGCTATAATGAAGCAATGGCTTCA ACCTCCAACAGGAGAGGTCCCCAGTT	225	57
Colicin M	Mf Mr	CCAGCAACCCTCTCACATTGCAG CCAGAAAACATCGCCCCGAGCC	556	68
Colicin V	Vf Vr	CACGCCCTGAAGCACCACCA CCGTTTTCCAAGCGGACCCC	400	68
Colicin 1a 1b	1abf 1abr	GCACAACAGGCCCGTCTGCTC CACCTTCCACATCCTCTGTCACC	385	68
Colicin E2, E3, E3a, E4, E5, E6, E7, E8, E9	Mixf Mixr	CGACAGGCTAAAGCTGTTCAGGT TGCAGCAGCATCAAATGCAGCCT	219	60
Colicin U, Y	UYf UYr	GTGAACGGACAGAAACCCGCC CAATCTGTCTGACAGCCTCTCCC	243	68
ColicinB, D, D157	BDf BDr	TCGCTCCATCCATGCCTCCG CCATCCCGACCAGTCTCCCTC	138	68
Colicin E1	E1f E1r	ACGGGAGTGGCTCTGGCGG CTCTTTACGTCGTTGTTCTGCTTCCTG	389	68
Colicin 5, 10, K	510Kf 510Kr	AAAGCTGAACTGGCGAAGGC CAACTCATCATCCCCTATGTAAGAAG	803	60

2.3 Results

2.3.1 Isolation of *E. coli* strains from rat faecal matter:

On the Hichrome coliform agar plates, rat faecal samples contained *E. coli* (4.79±1.204) $\times 10^8$ (Cfu gm-1 wet faecal samples) after weaning and (2.32±1.021)$\times 10^6$ (Cfu gm-1 wet faecal samples) at adults stage.

2.3.2 Screening of *E. coli* for antimicrobial activity:

In primary screening the antimicrobial activity of pure isolates were determined by agar spot method against *E. coli* (DH5α and BL21) on Luria agar (LA). A total of 47 of 288 isolates of *E. coli* produced zone of inhibition against *E. coli* DH5α and BL21 strains. Secondary screening was performed by induction of 47 isolates with Mitomycin C. Antimicrobial activity of the culture filtrates against the test organisms *Escherichia coli*, *Enterobacter asburiae*, *Klebisella* spp., *Staphylococcus aureus*, *Salmonella typhi* and *Salmonella abony* is shown in **Table 2.3** and **Fig. 2.1**. Culture filtrates which showed antimicrobial activity towards pathogens were treated with Proteinase K or Protease (trypsin) which resulted in lost of antimicrobial effect thus suggesting that the antimicrobial compound is a protein Fig. Sixteen out of 47 isolates showed inhibitory activity against different pathogens where as other isolates only inhibited E. coli DH5alpha.

Fig. 2.1: Antimicrobial activity in *E. coli* isolates the presence of Mitomycin C induction

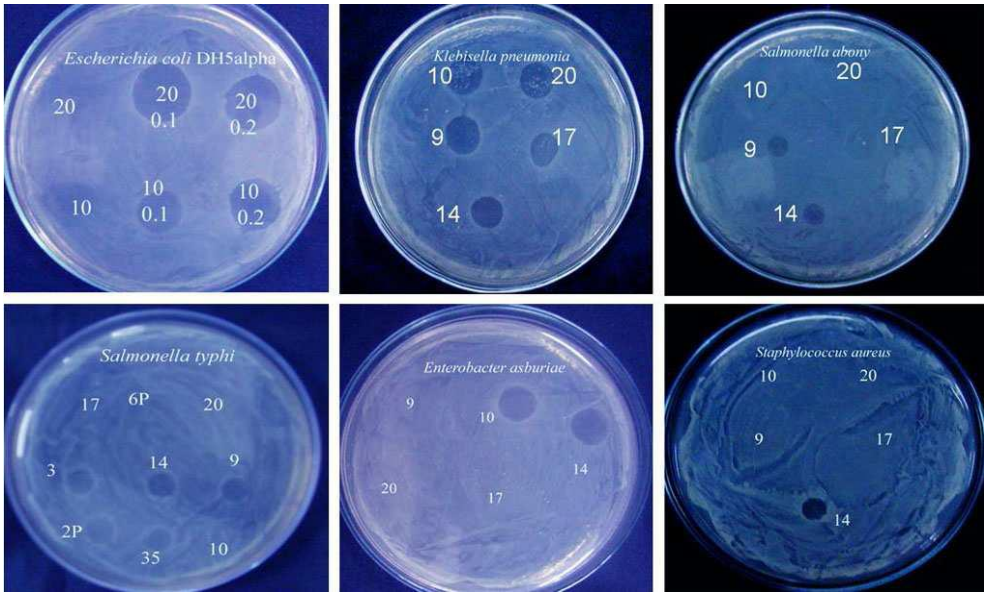
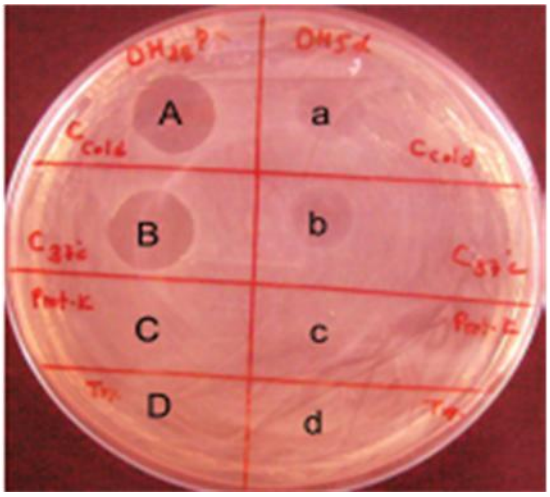


Table 2.3: Antimicrobial activity of *E. coli* against enteropathogens

***E. coli* culture No**	**Enteropathogens**
10,14, 14P	*Enterobacter asburiae*
9, 10,14,17,20,14P	*Klebsiella pneumoniae*
3,9,14,35,44,2P,8P	*Salmonella typhi*
10,14,16,14P	*Staphylococcus aureus*
9,14, 2P,8P	*Salmonella abony*

Fig 2.2 Loss of Antimicrobial activity upon treatment of Trypsin and ProteinaseK



A. Induced supernatent at cold condition
B. Induced supernatent incubated at 37°C
C. Proteinase K treatment
D. Trypsin treatment
a. Uninduced supernatant at cold condition
b. Uninduced supernatant at 37°C
c. Proteinase K treatment
d. Trypsin treatment

2.3.3 Antibiotic susceptibility of rat faecal *E. coli* isolates:

Most isolates did not show multi-drug resistance and the isolates which showed low multiple resistances were eliminated in initial screening with commercial antibiotics (data not shown). 16 isolates were finally screened by using commercial antibiotics discs (HiMedia) **Table 2.4**.

Table 2.4: Antibiotic susceptibility pattern of *E. coli* isolates (Concentration of antibiotic in µg/ml)

Culture No	*A*	*G*	*Ac*	*Tb*	*Co*	*Ce*	*Na*	*Nt*	*Nf*	*Ak*	*Cf*	*Ca*	*Of*	*T*	*Cu*
	10	**10**	**30**	**10**	**25**	**30**	**30**	**30**	**300**	**30**	**5**	**30**	**5**	**25**	**30**
3	S	S	I	S	S	S	S	S	S	S	S	S	S	S	S
6	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
9	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
10	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
14	S	S	I	S	S	S	S	S	S	S	S	S	S	S	S
16	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
17	S	S	**R**	S	S	S	S	S	I	S	S	S	S	S	S
19	**R**	S	I	S	S	S	S	S	S	S	S	S	S	S	S
20	S	S	I	S	S	S	S	S	I	S	S	S	S	S	S
21	S	S	S	S	S	S	S	S	I	S	S	S	S	S	S
35	I	S	I	S	S	S	S	S	S	S	S	S	S	S	S
44	S	S	I	S	S	S	S	S	**R**	S	S	S	S	S	S
45	S	S	S	S	S	S	S	S	I	S	S	S	S	S	S
2P	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8P	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S
14P	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

2.3.4. Acid tolerance assay:

Acid tolerance assay was performed at different pH 1.0, 2.0, and 3.0 for the eight antibiotic sensitive strains. Isolates *E. coli* strains 10, 20 and 16 showed higher acid tolerance whereas *E. coli* strains 3, 44, 45, 14 and 17 showed poor acid tolerance at pH 2.0. But at pH 3.0, all the isolates showed good acid tolerance up to 6 h. However, none of the isolates showed acid tolerance at pH 1.0 (**Table 2.5**).

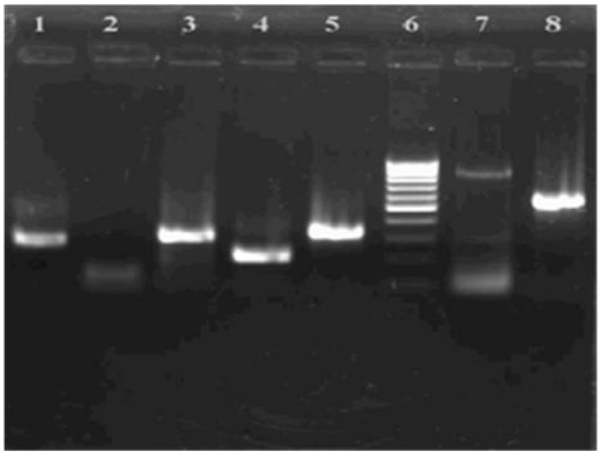
Table. 2.5: Acid tolerance tests for *E. coli* isolates (Survival percentage)

	pH 1.0	pH 2.0			pH 3.0		
STRAIN	**2h**	**2h**	**4h**	**6h**	**2h**	**4h**	**6h**
***E. coli* 17**	**0.00±.00**	**0.74 ± 0.15**	**0.09 ± 0.03**	**0.01 ± 0.00**	**71.95 ± 2.47**	**61.50 ± 2.12**	**39.40 ± 3.68**
***E. coli* 14**	**0.00±.00**	**2.26 ± 0.21**	**0.60 ± 0.14**	**0.00 ± 0.00**	**35.50 ± 4.95**	**19.80 ± 3.11**	**11.95 ± 0.92**
***E. coli* 45**	**0.00±.00**	**0.22 ± 0.01**	**0.15 ± 0.01**	**0.06 ± 0.01**	**46.50 ± 6.36**	**38.65 ± 1.06**	**35.55 ± 0.21**
***E. coli* 44**	**0.00±.00**	**2.1 ± 0.14**	**1.18 ± 0.03**	**0.18 ± 0.01**	**67.00 ± 5.66**	**58.15 ± 3.04**	**14.80 ± 1.13**
***E. coli* 3**	**0.00±.00**	**0.415 ± 0.01**	**0.35 ± 0.00**	**0.09 ± 0.01**	**68.25 ± 2.52**	**56.00 ± 8.49**	**43.13 ± 1.28**
***E. coli* 10**	**0.00±.00**	**8.35 ± 0.35**	**7.35 ± 0.21**	**6.80 ± 0.28**	**74.50 ± 4.95**	**25.15 ± 0.49**	**21.40 ± 1.41**
***E. coli* 20**	**0.00±.00**	**0.215 ± 0.01**	**0.13 ± 0.04**	**0.00 ± 0.00**	**41.75 ± 2.76**	**35.53 ± 0.75**	**31.15 ± 1.63**
***E. coli* 16**	**0.00±.00**	**41.35 ± 0.78**	**15.70 ± 2.40**	**0.06 ± 0.01**	**76.20 ± 1.13**	**74.10 ± 1.84**	**45.20 ± 0.85**

2.3.5. Detection of pathogenic strains by Multiplex PCR of indicator genes.

For 7 indicator genes: *aggR, est, aspU, CVD432, elt, eae* and *stx*, positive control were obtained. (**Fig. 2.3**). The sizes of the amplicons were similar to the reported pathogenic genes. Hence, the primers were used to determine the presence of the pathogenic genes in the *E. coli* isolates. No amplicons were obtained from the genomic DNA of all the *E. coli* isolates.

Fig. 2.3: PCR foridentification of pathogenic genes from activated sludge samples

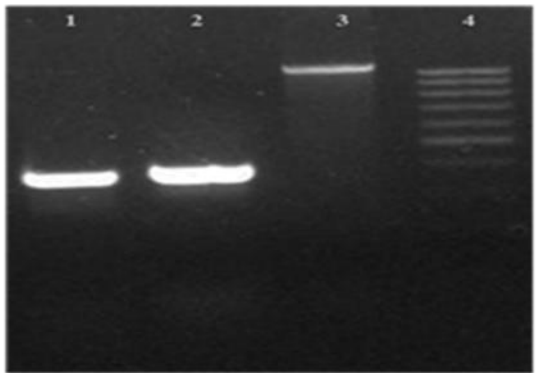


Lane 1-aggR
Lane 2-AL
Lane 3-aspU
Lane 4-Eagg
Lane 5-LT
Lane 6 – 100bp ladder
Lane 7 – SK amplicon
Lane 8 – VT

2.3.6. Polymerase chain reaction (PCR) for Identification of colicin gene in *E. coli* isolates

Isolates 17 contained only E1 type; isolates 3 9, 10,35, 44, 8P and 14P contained E1 and 1a/1b type showed in **Fig. 2.4**; isolates 16 and 2P contain E1, 1a/1b and Emix type; and isolates 14 and 20 contained E1, 1a/1b and B/D type colicins.

Fig. 2.4 PCR amplification of colicin genes



Lane 1:.1a/1b colicin 385bp
Lane 2: E1 colicin 389 bp,
Lane 3: E1 and 1a colicin 1.1kb
Lane 4: 100bp DNA ladder

2.4 DISCUSSION

*E. coli c*ount in the rat faecal matter decreased from weaning to the adult stage by about hundred fold. Variation in the abundance and diversity of microflora has been found to occur during the development of animals. In the initial stages the microflora is found to be more diverse and decrease during the transition to the adult stage (Inoue *et al*., 2005). In addition, the composition of the intestinal microflora is influenced by nature of the diet, antibiotic treatment and infection of exogenous opportunistic pathogens (Jernberg *et al*., 2005; Gostner *et al*., 2006). The results of the present study demonstrated that some of the *E. coli* strains (isolated of faecal samples) showed antimicrobial activity against the members of *Enterobacteriaceae* family *viz*. *Enterobacter asburiae*, *Klebisella* spp., *Staphylococcus aureus*, *Salmonella typhi*, and *Salmonella abony*. These isolates were susceptible to the most of antibiotics tested and low resistance was observed in some cases which were eliminated in the screening. In feaces, population of antibiotic resistant and sensitive microorganisms varies. (Mubita *et al*., 2008; Schierack *et al*., 2009). Multiplex PCR studies demonstrated that these *E. coli* strains do not contain any pathogenic traits. Antibiotic sensitivity and non-pathogenic traits are essential for probiotic organisms.

Antimicrobial activity of these *E. coli* strains was enhanced by Mitomycin C induction. Mitomycin C is known to induce the production of colicins (Cascale *et al*., 2007). Antimicrobial activity of *E. coli* strains 10, 14, and 14P demonstrated broad host range. Majority of the *E. coli* strains contained colicins E1 and 1a/1b, in contrast to the combination of Microcin H47 with Microcin M was reported (Gordon at el., 2006) These colicins belong to pore forming type. Interestingly, 4 *E. coli* strains along with these pore forming colicins produced other colicins. Isolates 16 and 2P possessed E mix which belong to nuclease type, whereas isolates 14 and 20 had B/D types, which are translation blockers.

Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach. Preliminary experiments showed that these colicin producing *E. coli* strains also had good but variable acid tolerance at pH 3.0. *E. coli* can survive at a

pH as low as 2.0 upon induction of acid resistant genes (Hersh *et al*., 1996; Lin *et al*., 1996). Thus, these *E. coli* strains have great potential as probiotic bacteria against GI diseases caused by enteropathogens.

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 (~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 (~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, 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*., 1995b; Jones et al., 2007).

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

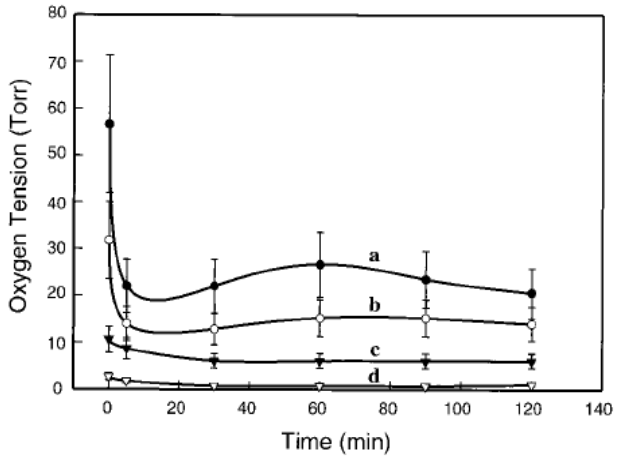
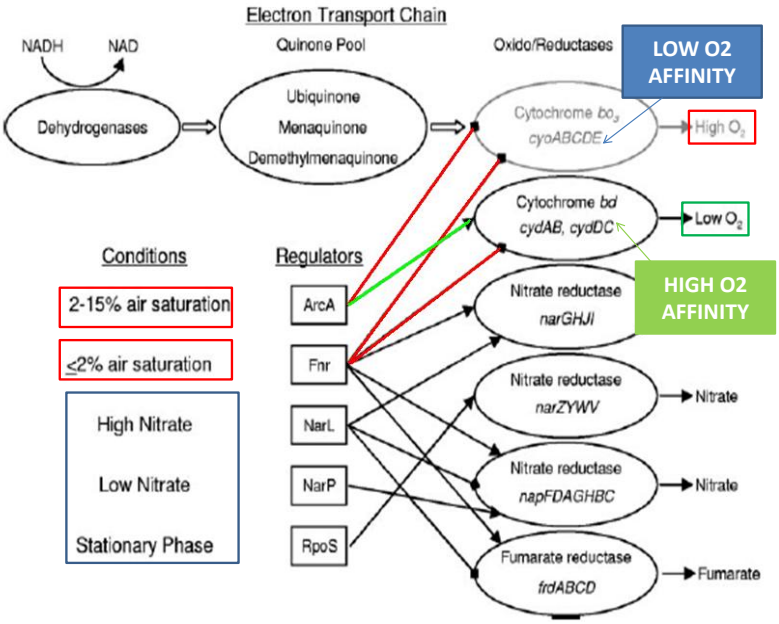
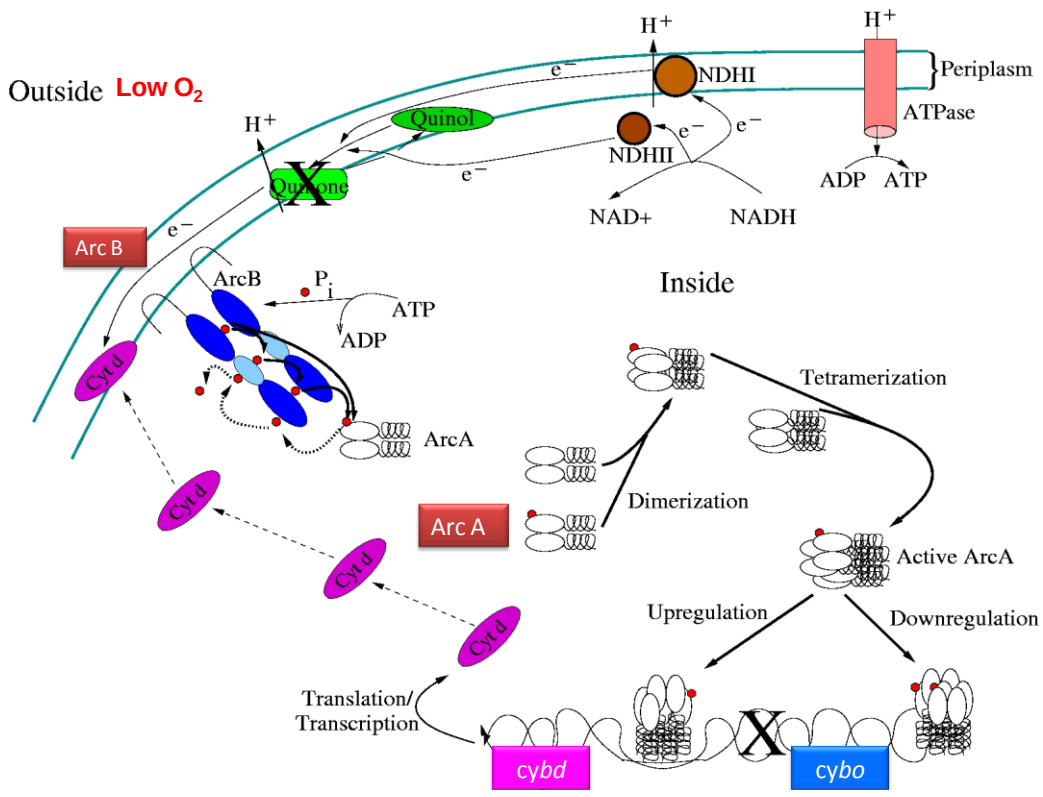


Fig. 3.2 Overview of mechanism of Arc/Fnr system (Jones *et al.*, 2007)



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).

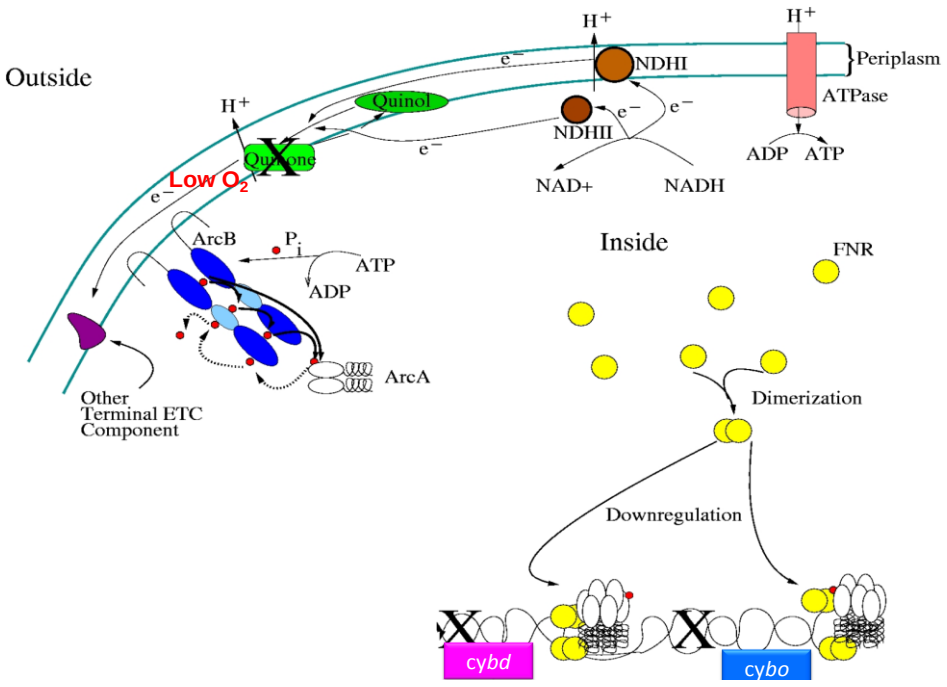
Fig. 3.3 Mechanism of ARC system (Peekhaus and Conway, 1998)



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; Isarankura-Na- Ayudhya *et al*., 2010). 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 culture 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 ºC). 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 spreading of the feacal flora (Schultza *et al*., 2005; Jones *et al*., 2007) to findout the total count of pUC8-16gfp tagged E. coli.

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

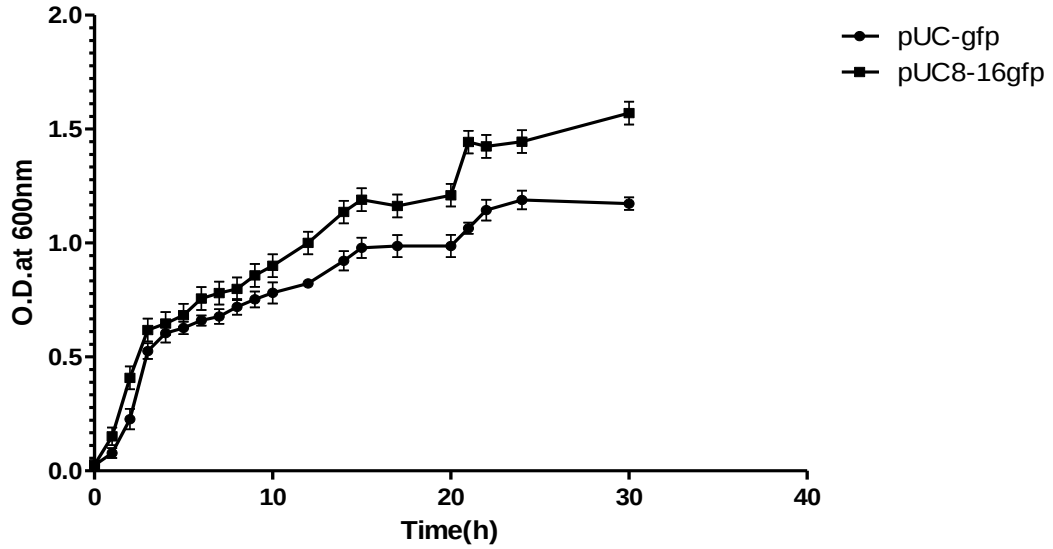


Fig. 3. 6A. SDS-PAGE analysis of *E. coli* BL21 pUC8-16*gfp* lysate under aerobic condition

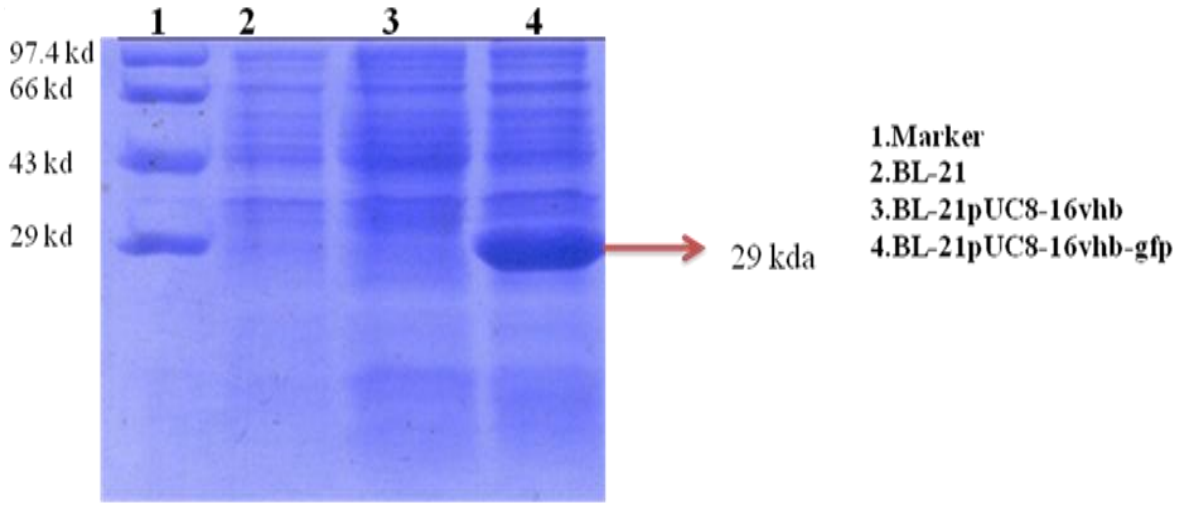
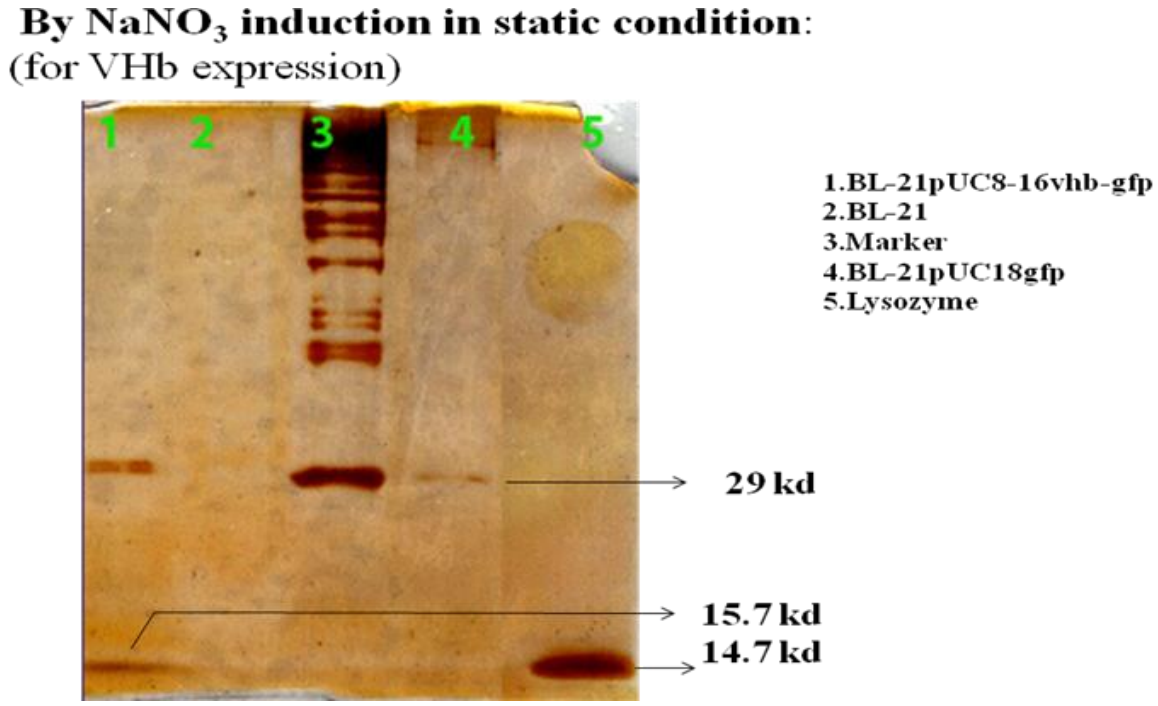


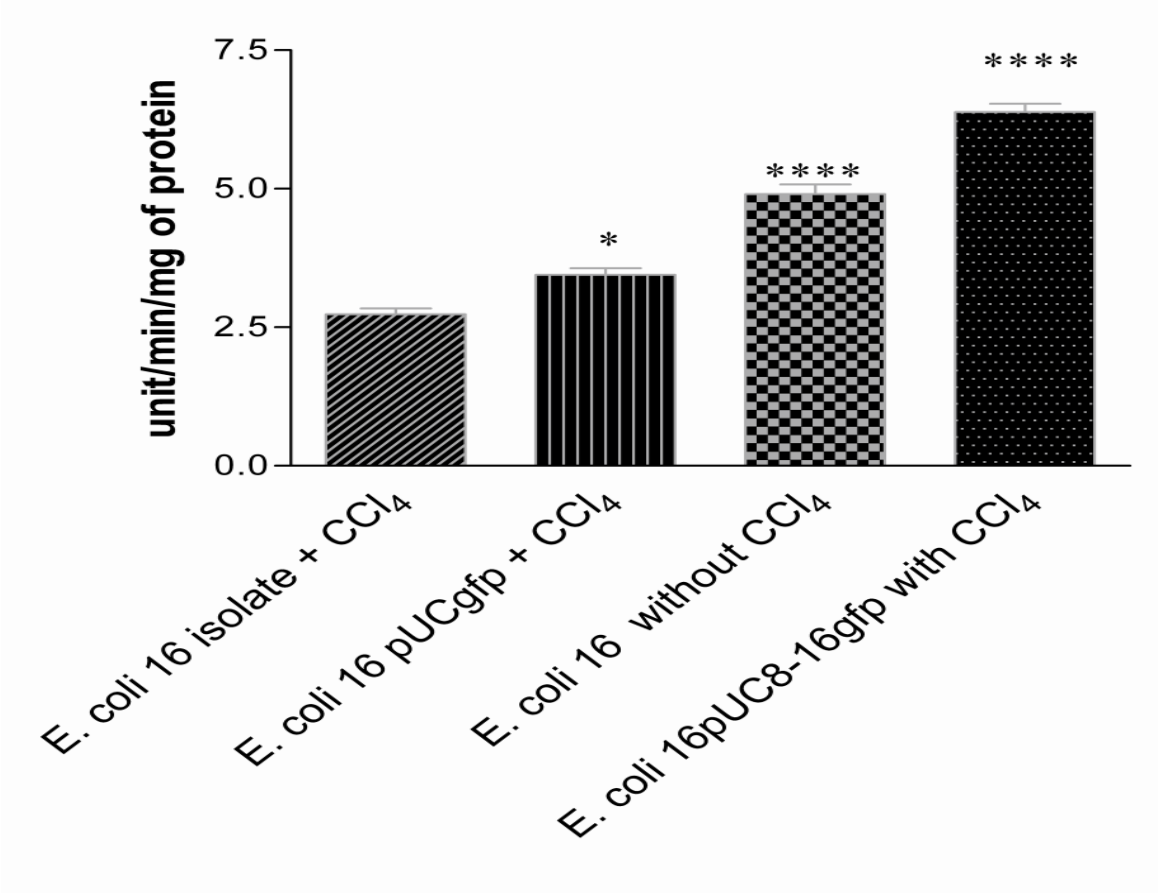
Fig. 3.6B. SDS-PAGE analysis for expression of *E. coli* BL21 pUC8-16*gfp* by $NaNO_3$ induction condition.



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 21st day, the feacal cultures for *E. coli* 16 (pUC-*gfp*) were 100 times reduced compared to *E coli* 16 (pUC8-16*gfp*). After 22nd to 24th 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 70th 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 48th 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.

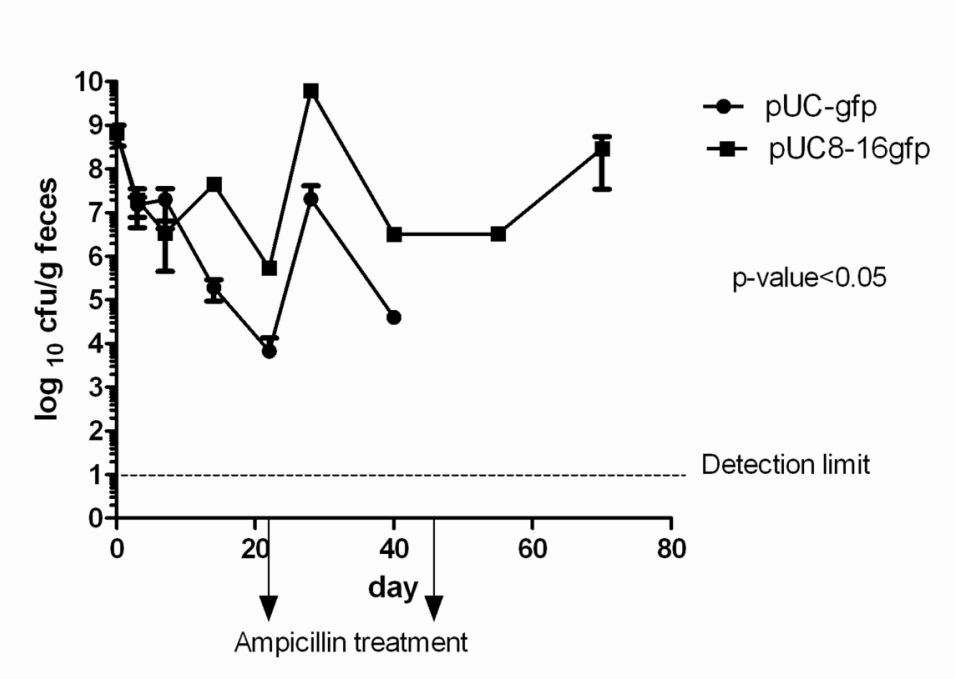


Fig. 3.8 B. Antimicrobial activity of *E. coli* 16 transformed with pUC8-16*gfp* plasmid in feacal samples of Charles Foster rats

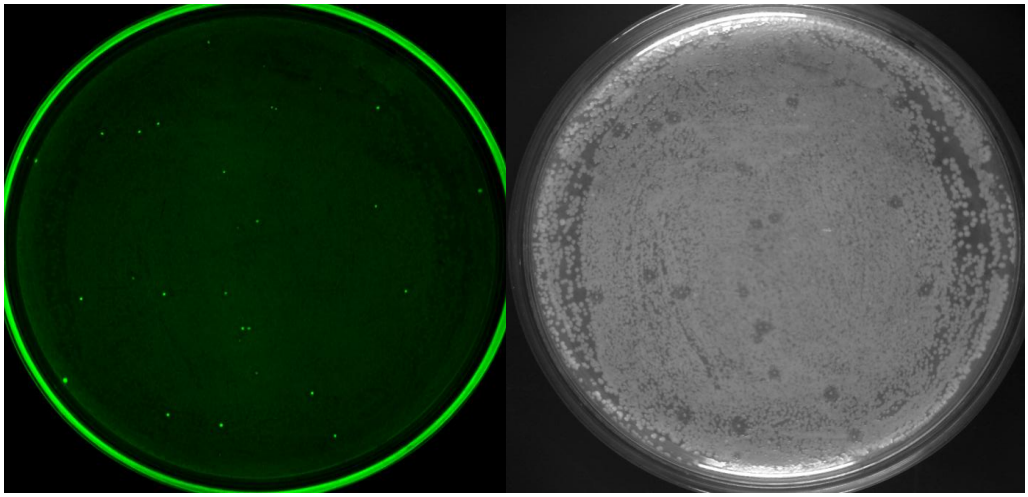
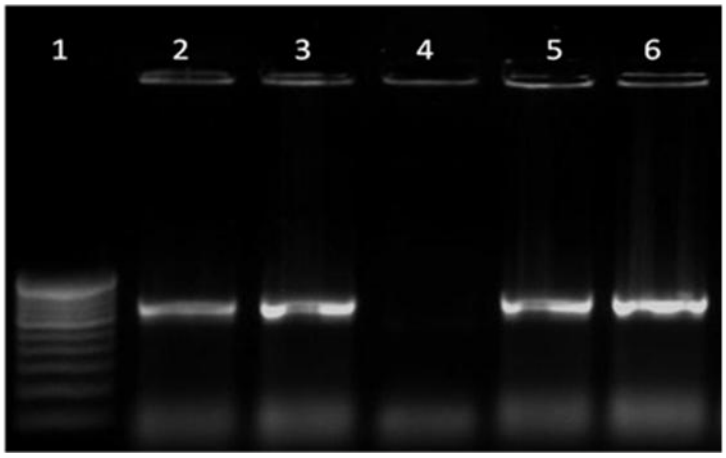


Fig. 3.8 C. Colony PCR amplification of *vgb* gene.



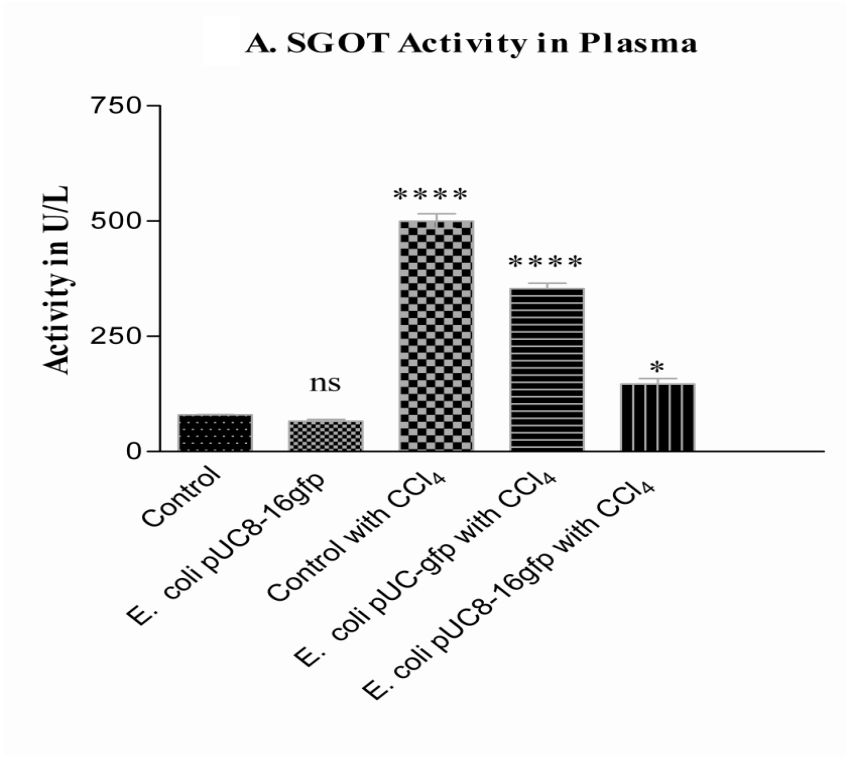
Lane 1: 100bp ladder
Lane 2: 714 bp
Lane 3 5: 714 bp
Lane 4: negative control
Lane 5: 714 bp
Lane 6: plasmid as positive control showed PCR.

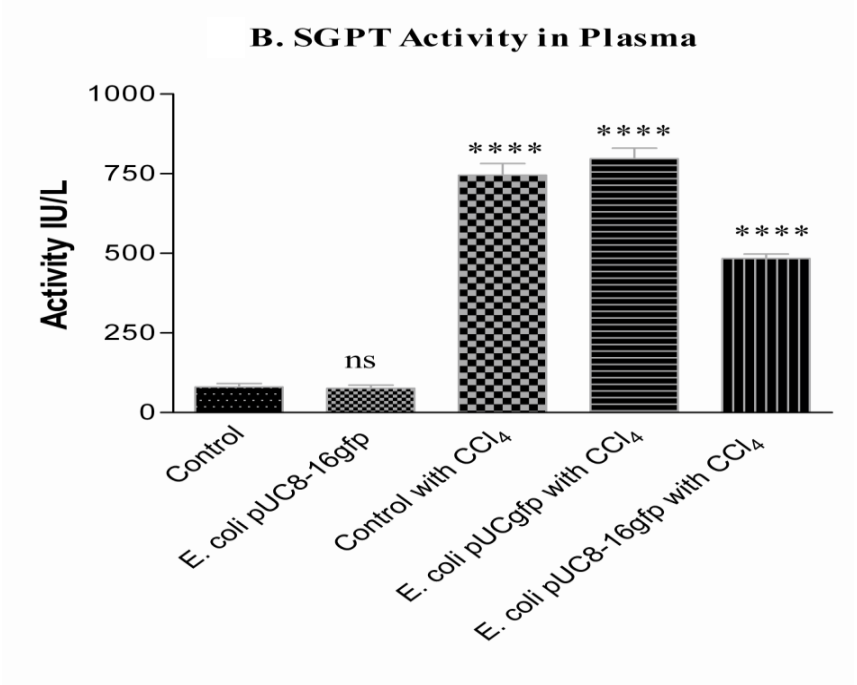
3.3.2.2 Effects of probiotic *E. coli* 16 (pUC8-16*gfp*) harboring *vgb* gene on liver function under CCl_4 induced oxidative stress.

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.

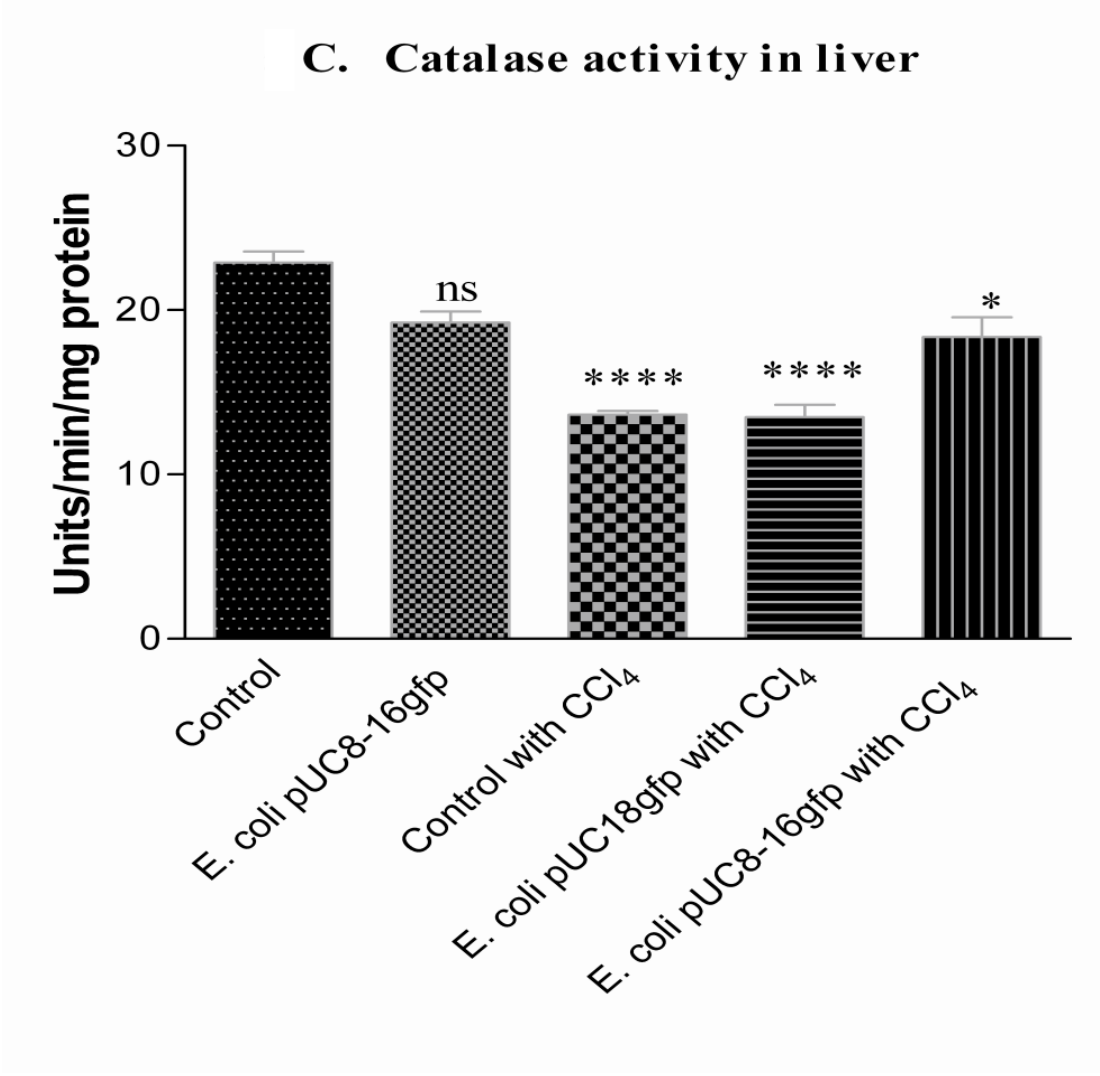


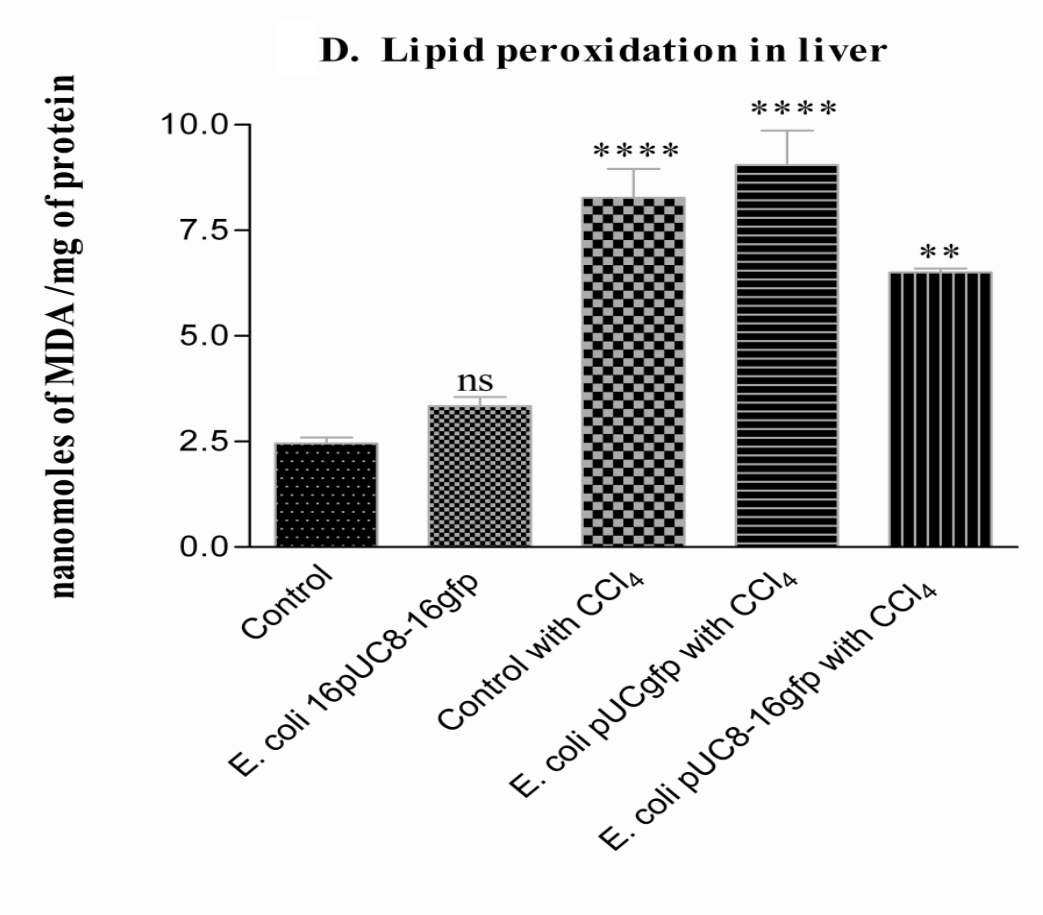


Values are expressed as mean ± SD of three rats in each group. P< 0.05 (ANOVA).

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**).

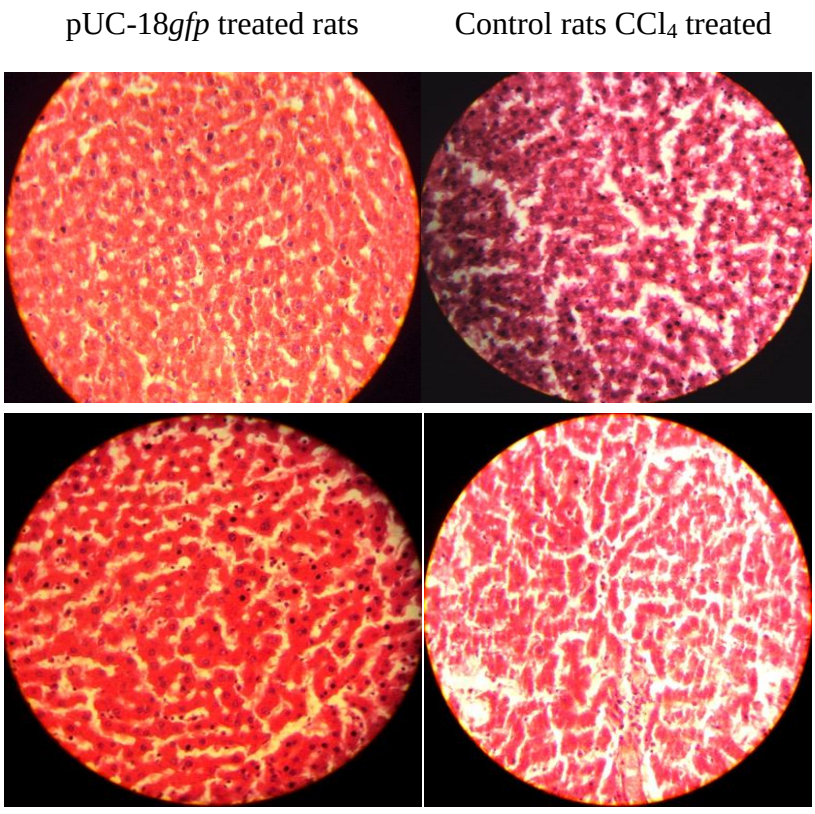




3.3.2.2.3 Microscopic examination of liver.

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**).

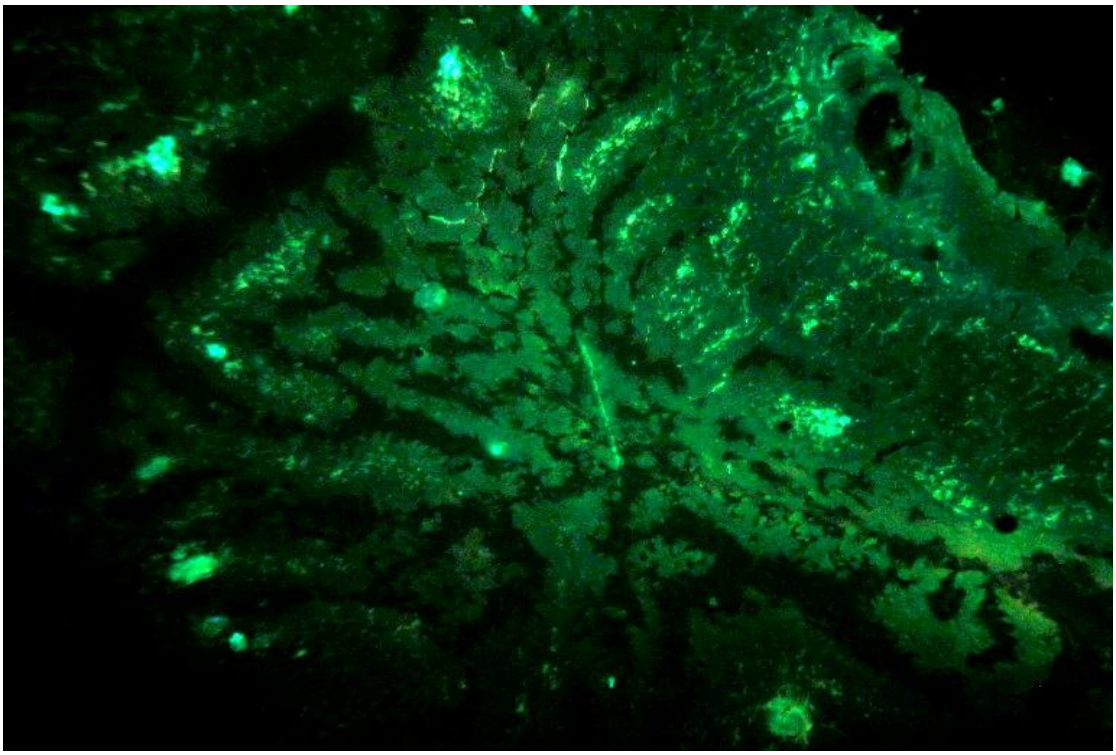
Fig. 3.10 Effect of probiotic *E. coli* harboring *vgb* gene on CCL_4-induced histopathological changes in rat liver. (A) Photomicrograph of liver from control rat with probiotic *E. coli* (pUC8-16*gfp*), (B) photomicrograph of liver treated with CCL_4, (C) photomicrograph of liver treated with CCL_4 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. $CC1_3OO^{\cdot}$ formed from O_2^- and $CC1_4$ 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). 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 in 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.

CHAPTER 4

Molecular Fingerprinting of the feacal microbiota in relation to high fructose induced metabolic disorder.

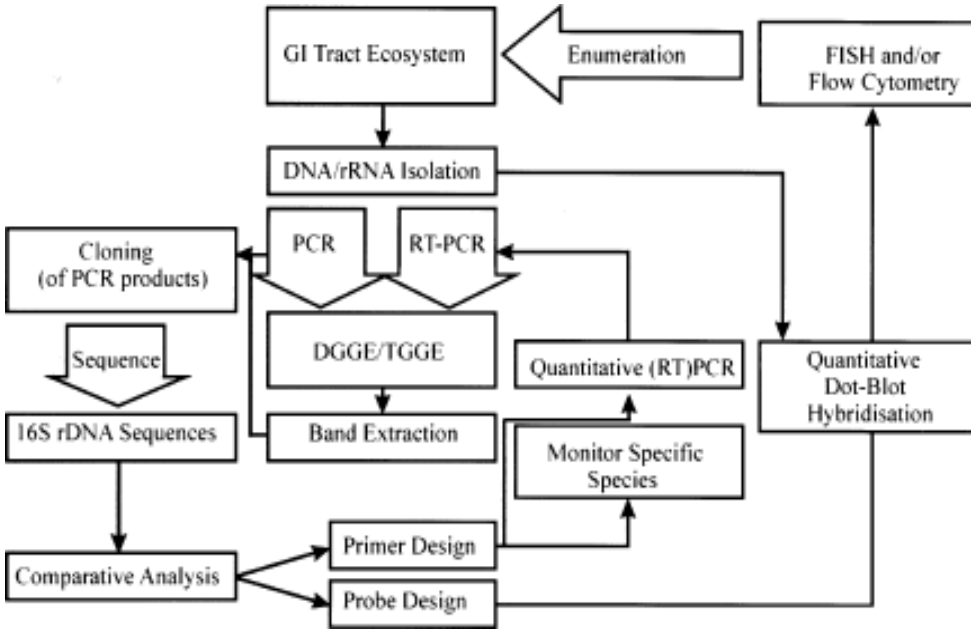
4.1 Introduction

The mammalian body harbours large microbial populations on the skin, in the oral cavity and in the genital and GI tracts. The number of bacteria is thought to exceed the number of mammalian cells by as much as a factor of 10, and a majority of bacteria are present in the GI tracts (Backhed *et al*., 2005). Our knowledge and understanding about gene and disease is now focused towards microbial community that reside in human gut (Yang *et al*., 2009; Benson *et al*., 2010). The microbial community reside in human gut manipulates gut physiology and thereby influences the health. The change of human gut microbial community is associated with obesity, diabetes, hypertension, and gastrointestinal disorder. Therefore, gut bacteria has become very significant areas of research especially for dietary related metabolic disorders. The environment in various sections of the GI tracts is constantly changing; this is reflected by the variations in the composition and population of bacterial community in various sections of the human and infants' GI tracts, and faeces (Gibson *et al*. 1998; Mueller *et al*., 2006; Vaishampayan *et al*., 2010). More than 90% of bacteria present in gut are unculturable. A cultural technique only contributes 10% of entire gut microbiota (Sghir *et al.,* 1999). Cultural approach contributes significantly in understanding the microbial diversity but is limited by lack of precision, low level of reproducibility and labour intensive and thus limiting the effectiveness for analysing the large number of community sample for diversity study. *The development of molecular techniques to investigate microbial diversity has provided the microbiologist with a vast array of new techniques to investigate human intestinal microflora. Various molecular approaches are available to analyse the microbial diversity.* (Vaughan *et al.,* 2000; Keeley et. al., 2009) **(Fig. 4.1; Table 4.1).**

Table 4.1: Potential use and drawbacks of methods for analysis of complex microbial communities (Vaughan *et al*., 2000; Keeley *et al*., 2009).

Method	Uses	Drawbacks
Culturing	Isolation; "The ideal"	Not representative; slow
16S rDNA sequencing	Identification	Large scale cloning is laborious
DGGE/TGGE	Rapid comparative analysis; identification by band extraction; detection of specific groups	Semi-quantitative
T-RFLP	T-RFLP Rapid comparative analysis; very sensitive; potential for high throughput	Semi-quantitative; identification only possible with clone library
SSCP	Rapid comparative analysis	Semi-quantitative; identification only possible with clone library
FISH	Detection; enumeration; comparative analysis possible with automation	Requires probe design; laborious without automation
Dot-blot hybridisation	Detection; estimates relative abundance	Requires probe design; laborious
FISH/Flow cytometry	Enumeration; potential for high throughput	Under development

Fig. 4.1: Flow chart of molecular approaches used to analyse the human intestinal microbial community (Vaughan *et al.*, 2000).



Metagenomic approach is a comprehensive study of nucleotide sequence, structure, regulation, and function, providing a picture of the dynamics of complex microbial communities (Tringe *et al*, 2005). This approach can identify the diversity, but not the relative numbers, of each species residing in that particular environment. Metagenomic approaches will provide a wealth of sequence information from single ecosystems, but they are not suitable for high-throughput monitoring. Denaturing gradient gel electrophoresis (DGGE) and related fingerprinting techniques have proven their power in comparing and monitoring ecosystems at the 16S rRNA gene or functional gene level (Ercolini, 2004; Licht *et al*., 2006). DGGE and Temperature gradient gel electrophoresis (TGGE) are gel-electrophoretic separation procedures for double stranded DNA's of equal size but with different base-pair composition or sequence (Muyzer and Smalla, 1998). In principle, the methods are sensitive enough to separate DNA's on the basis of single point mutations (Sheffield *et al*., 1989).

The composition of the diet influences the bacterial flora of the GIT. Diet and frequency of feeding influences the distribution of bacteria within the GIT of both man and animals (Sekirov *et al*., 2010**).** Amylase resistant starch (ARS) that is low digestibility starches alters the intestinal microflora of rats (Martínez-Puig *et al*., 2007). The mucosa associated flora of the large intestine is important in determining mucosal function. Microbial population on the mucosal surface depend on the prebiotic carbohydrates oligofructose and inulin (Langlands *et al*., 2004). The dietary analysis method provides a new tool for establishing relationships between diet and disease and indicates a potentially therapeutic diet for various disease i.e. UC (ulcerative colitis), diarrhoea, obesity and many gastrointestinal disorder (Elizabeth *et al*, 2005).

Dietary sugar such as fructose and sucrose is readily absorbed and rapidly metabolized by human liver. In 1977, average intake of fructose sugar in united nation was amount approximately 37 grams per day, largely from fresh fruits (Park and Yatley, 1993). Westernization of diet has resulted in significant increase in added fructose, leading to typical daily consumptions amounting to ≥ 100 grams of fructose per day (Basciano *et al*., 2005). The exposure of the liver to such large quantities of fructose leads to rapid stimulation of lipogenesis and triglycerides (TG) accumulation (**Fig 4.2**), which in turn contributes to reduced insulin sensitivity and hepatic insulin resistance/glucose intolerance. Intake of nutritive sweetener above 25% of total energy consumed will cause hypertriglycridemia and gastrointestinal symptoms (Tappy and Kim, 2010). The long term effect of high fructose diet can include changes in digestion, absorption, apetite and hepatic metabolism leading to development of various metabolic disorders such as diabetes, obesity and cardiovascular disease.

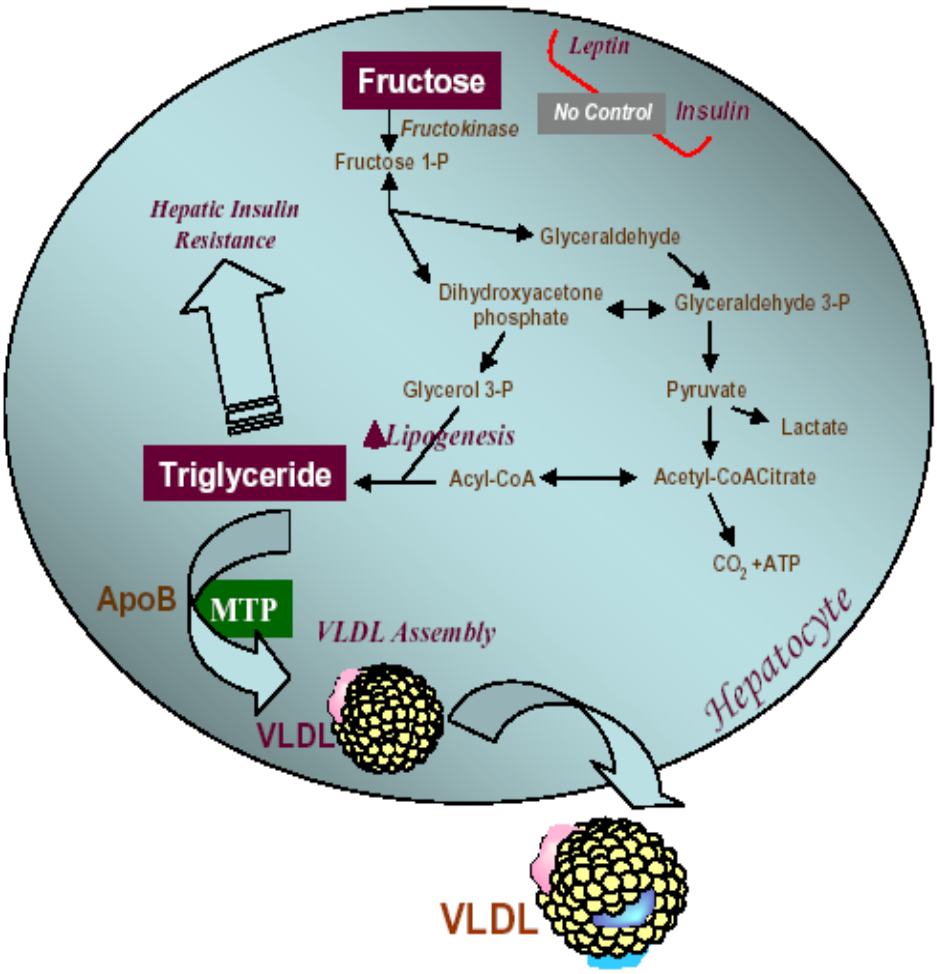


Fig. 4.2: Hepatic fructose metabolism: A highly lipogenic pathway (Basciano *et al.*, 2005).

In this chapter the relationship between different dietary sugar and intestinal microbial community profiles was investigate by using DGGE.

4.2 Materials and Methods

4.2.1 Animals and treatment:

Male Charles Foster rats (body weight 200-250 g) were used for the study. They were allowed *ad libitum* access to water and food. The animals were cared in accordance with principles and guidelines of the Institutional Animal Ethics Committee (IAEC), Department of Biochemistry, M. S. University of Baroda,Gujarat, India. The experimental protocol was approved by the IAEC.

4.2.2 Experimental groups:

The animals were divided into four groups of six rats each. Group 1, control animals (control), received the control diet containing 61 per cent starch and tap water *ad libitum* [61% starch, 20% casein, 0.7% methionine, 5% groundnut oil, 9.7% wheat bran and 3.5% salt mixture (The mineral mix in a kg contained $MgSO_4$ $7H_2O$-30.5 g; NaCl-65.2 g; KCl-105.7 g; KH_2PO_4-200.2 g; $MgCO_3$ g, -3.65 g Mg $(OH)_2$. $3H_2O$-38.8 g; $FeC_6H_5O_7.5H_2O$-40.0 g; $CaCO_3$- 512.4 g; KI-0.8 g; NaF-0.9 g; $CuSO_4.5H_2O$-1.4 g; $MnSO_4$-0.4 and $CONH_3$ -0.05 g) and water *ad libitum*, 0.2 ml of vitamin mixture (The vitamin mix in a kg contained vitamin A concentrate I.P., 2500 I.U.; vitamin D3 cholecalciferol, 200 I.U.; thiamine hydrochloride, 0.5 mg; riboflavin, 0.5 mg; pyridoxin, 0.5 mg; sodium pantothenate, 1.5 mg; nicotinamide, 5 mg; ascorbic acid, 25 mg multivitamin tablets, Piramal healthcare Ltd., Mumbai, India) was added per kg feed. Group 2, fructose-fed animals (Fructose), received the fructose enriched diet, which was similar in composition to the control diet (except for starch which was replaced by 61% fructose). Group 3, Control animals treated with streptomycin, received the starch diet and were allowed to drink 5gm/ litre streptomycin solution 15 days interval and other days allowed *ad libitum* access to water. Group 4, fructose-fed animals treated with streptomycin, received the fructose fed diet and were allowed to drink 5gm/ litre streptomycin solution 15 days interval and other days allowed *ad libitum* access to water. The diets were prepared fresh everyday based on the method of Cohen *et al.*, (1977). The animals were maintained in their respective groups for 90 days. Body weight changes were measured initially and in the end of treatment. At the end of the experimental period oral glucose tolerance test was carried after 12 h fasting (blood samples were collected by

retro-orbital plexus) and blood samples (separated plasma) were used for biochemical estimations.

4.2.3 Genomic DNA isolation from feacal samples.

The genomic DNA isolation from feacal sample of rats was done by using standard method of Zhang *et al*., (2006), with slight modification. Shortly, 1-1.5 g of freshly collected faeces of rat was dissolved by vigorous vortexing in 0.85% saline. Pellets were collected by centrifugation at 5000g. The pellets were washed with TE buffer, resuspended in 3ml TNE reagent and incubated at 55°C for 1-2 h after addition of 50 µl of proteinase-K. Supernatant was collected in fresh eppendorf containing 2 g starch powder, gently mixed by vortexing and incubated for 1 min at room temperature. 100 µl of 5M NaCl and 180 µl CTAB /NaCl solution were added to the supernatant collected after centrifugation and incubated for 10 min at 65°C. The mixture was extracted twice with equal volume of phenol / chloroform / isoamyl alcohol followed by once with Chloroform/isoamyl alcohol. Finally the supernatant was collected in a fresh tube and added 0.6 vol of isopropanol, mixed gently and incubated at room temperature for 1 h. Precipitate was collected by centrifugation followed by 70% ethanol wash. After complete removal of ethanol by air drying, the pellet was finally dissolved in 25 µl 10:1 TE buffer.

4.2.4 PCR amplification of V3 region of 16S rDNA from *feacal* matter.

The variable V3 region of 16S rDNA gene was amplified by PCR using the following primers complementary to conserved regions of the 16S rRNA genes correspond to positions
341 and 534 in *E. coli* (Muyzer *et al*., 1993). Forward primer contains 40 nucleotide GC-rich sequence which acts as a clamp.

Forward Primer: 5' CCTACGGGAGGCAGCAG 3'
Reverse Primer: 5' ATTACCGCGGCTGCTGG 3'
Forward primer GC-rich sequence:
5'CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCCTACGGGAG GCAGCAG 3'

A combination of Forward primer GC-rich sequence and reverse primer was used to amplify the 16S rDNA regions from DNA obtained from *feacal* samples.PCR cycling conditions used to amplify the 16S rDNA gene fragment were 96º C for 5 min, followed by 10 cycles of 94º C for 1 min, 61º C (reduced by 0.5º C each cycle) for a 45 s and 72º C for 1 min; 25 cycles of 94º C for 1.0 min, 55º C for 45 s and 72º C for 1.0 min; and a final extension at 72º C for 10 min.

4.2.5 Denaturing gradient gel electrophoresis (DGGE)

The Dcode universal mutation detection system (Bio-Rad Laboratories, Richmond, Calif.) was used for a DGGE analysis of the PCR products obtained faecal microbial community DNA. Electrophoresis was performed in a 0.8-mm polyacrylamide gel (10 % [wt/vol] acrylamide-bisacrylamide [37.5:1]) by using denaturant gradient from 40 to 60% (100% denaturant was 7 M urea plus 40% [wt/vol] formamide increasing in the direction of electrophoresis, were used. The gels were subjected to a constant 80 V for 12 h at 60°C, and after electrophoresis the gels were stained by silver staining method.

4.2.6 Silver staining

The silver staining was done by the standard method as described in Sambrook *et al* (2001).

4.2.7 Biochemical analyses in serum samples

Oral glucose tolerance test, total cholesterol, triglycerides, HDL and LDL cholesterol concentrations were measured.

4.2.8. 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 version 3.0 for Windows, Graph Pad Software,San Diego, California, USA.

4.3 Results

4.3.1 Microbiota of the rat faecal matter fed with starch and fructose in diet.

PCR amplifications of 16S rRNA gene fragments were obtained from total DNA extracted from faecal samples using specific primers as mentioned in material and method (4.2.4) (**Fig. 4.3**).

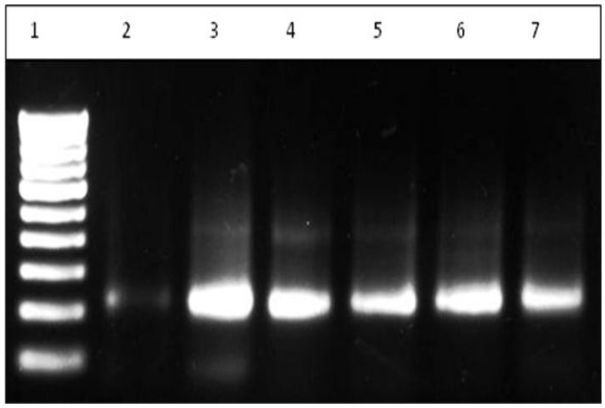
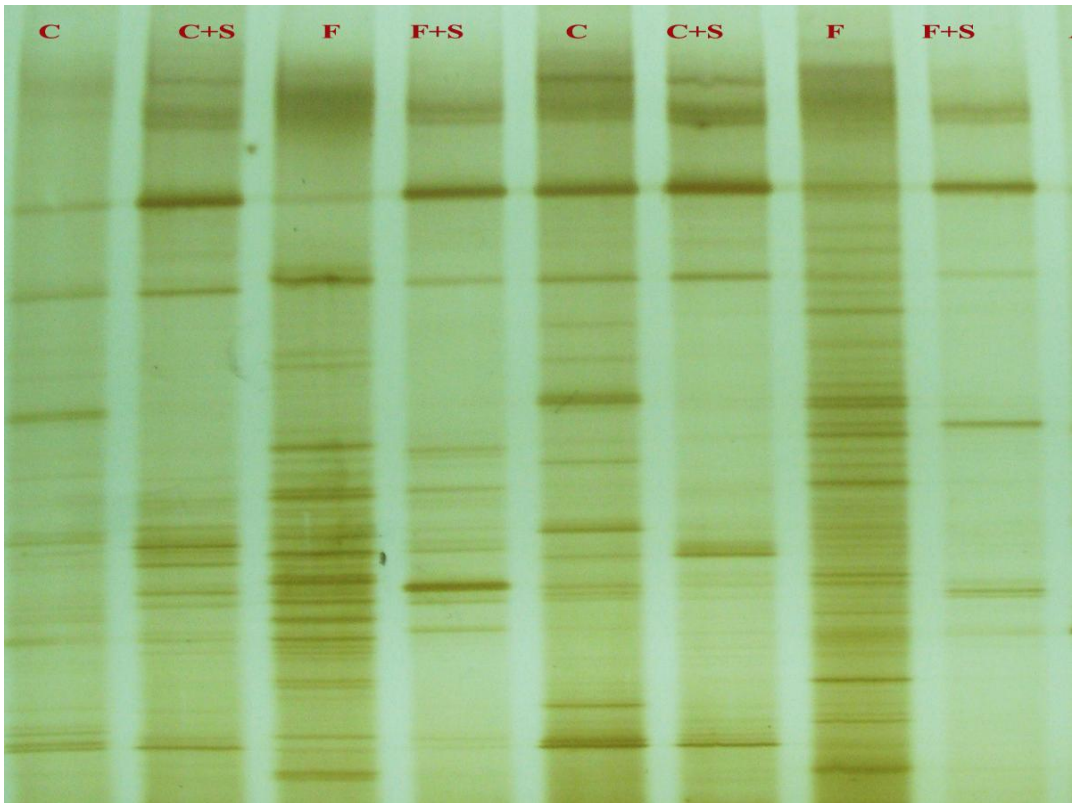


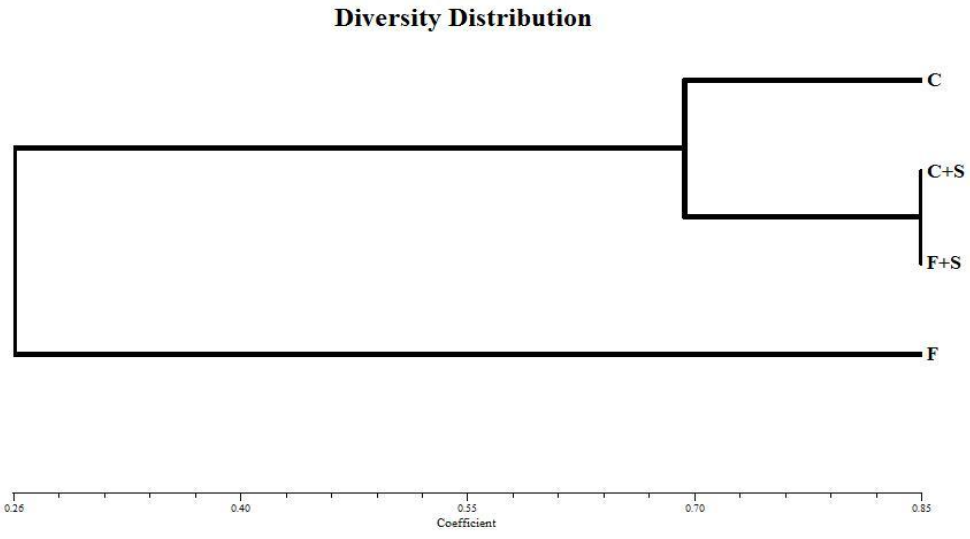
Fig. 4.3: 16S rRNA gene PCR of starch and fructose treated feacal microbial population. (2% agarose). Lane 1: 100 bp Ladder, Lane 2, 3, 4, 5, 6 and 7 PCR from feacal matter of different dietary group.

DGGE profiles revealed that faecal samples from animals belonging to the same dietary groups showed similar banding pattern but differed from other dietary groups (**Fig. 4.4**). The microbial profiles of rats fed on starch and fructose were entirely different but the profile of streptomycin treated groups was similar. Changes in the carbohydrate composition (starch fed and fructose fed rats) influenced the phylogenetic composition of the GI tract microbiota

Fig. 4.4: DGGE profile and dendrogram of faecal genomic DNA samples from rats receiving starch and fructose -base diet.



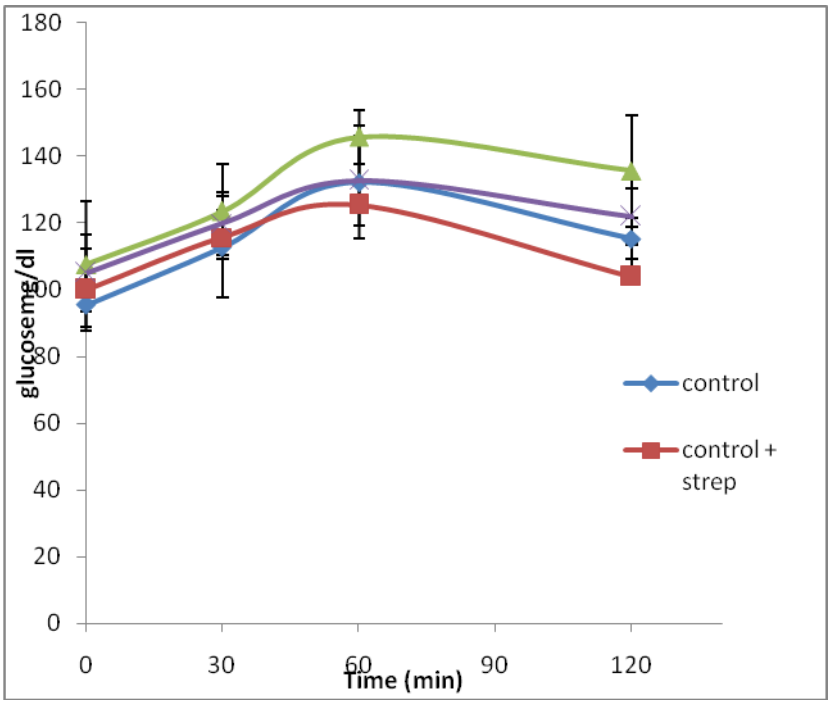
C= Control, C+S= Control+ Streptomycin, F= Fructose, F+S= Fructose + Streptomycin



4.3.2 Oral glucose tolerance

After 70 days on the 61% (w/w) sucrose rich dietary group, the rats developed severe glucose intolerance (**Fig. 4.5**). Compared to control there is significant increase in plasma glucose level in fructose (61%) fed groups.

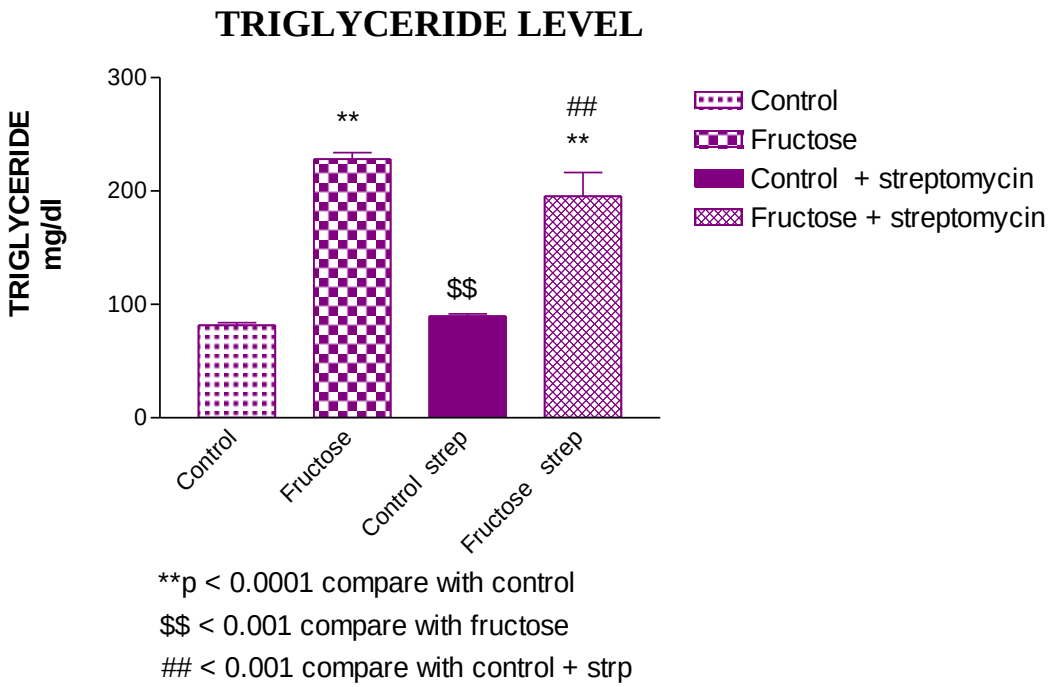
Fig. 4.5: Oral glucose tolerance tests in plasma samples of different groups of rat fed with Starch and Fructose.

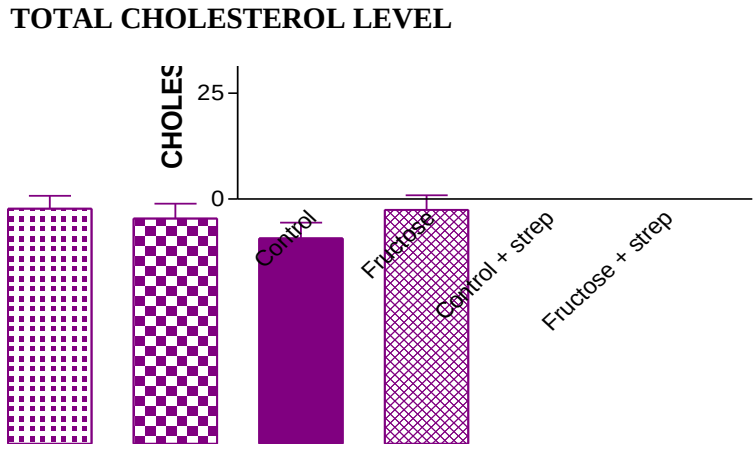


4.3.3 Serum lipid levels

Comparison of the effect of fructose and starch supplementation on the fasting serum lipid was estimated in rats (**Fig. 4.6**). TG was significantly increased 2.8 fold in fructose rich diet compared to control. Cholesterol levels were not changed in the entire diet group. HDL-Cholesterol was reduced to 0.76 fold in fructose rich diet compared to control. Similar trends were found when compared between control and fructose treat streptomycin groups.

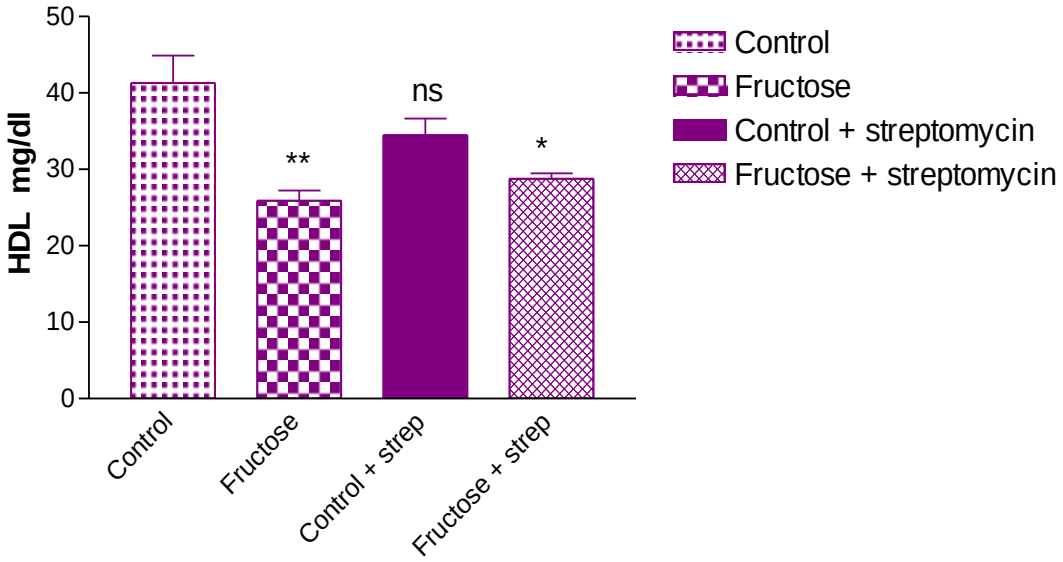
Fig. 4.6. Lipid profile in serum samples of different groups of rat fed with Starch and Fructose.





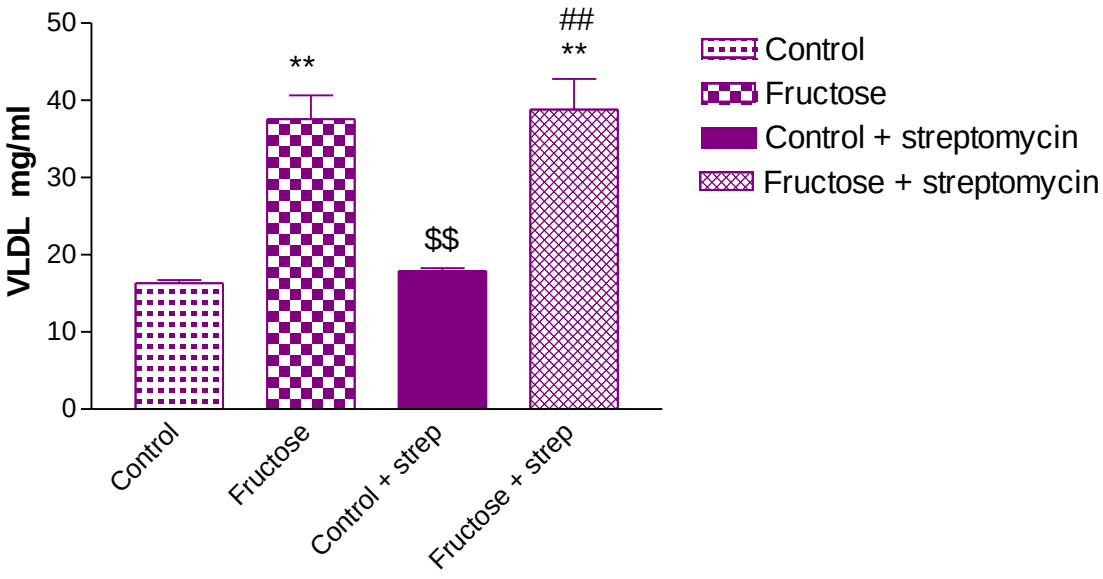
No significant change between groups , N = 6-8

HDL-CHOLESTEROL LEVEL



**P < 0.001 & *P < 0..01 compare to control N = 6-8
other group are non significant.

VLDL Level



$$ < 0.001 compare to fructose

< 0.001 compare to control + strp

**P < 0.001 & *P < 0.01 compare to control N= 6-8

4.4 Discussion

The worldwide increase in the incidence of metabolic syndrome correlates with marked increase in total fructose intake in the form of high-fructose corn syrup, beverage and table sugar. High fructose diet causes hypertension, insulin resistance and hyperlipidaemia (Johnson *et al*., 2007). The complex interrelationship between dietary components, gut microbiota composition and function and the human cardiometabolic phenotype are largely lacking (Diamant *et al*., 2011). However, recently various meta-omics-based approaches have been developed to unravelling the secrets of our gastrointestinal tract microbiota (Zoetendal *et al*., 2008). In this study, culture-independent approach based on the 16S rDNA sequence variability was used. DGGE profile showed that changes in the dietary sugar affected the phylogenetic composition of the faecal microbiota. Even though sucrose and starch are both easily digestible, they are not expected to reach the large intestine. The DGGE band patterns obtained indicated that these carbohydrates indeed affected the composition of bacteria in the large gut. Based on similarity and cluster analysis, starch and fructose treated dietary groups had different phylogenetic composition of the faecal microbiota. Recent reports mentioned about dietary change that influenced the gut microbial diversity (Cani *et al*., 2008; Turnbaugh *et al*., 2009; Faith *et al*., 2011, Wu *et al*., 2011). On the other hand, streptomycin treated groups did not show any variation.

High fructose diet also altered the clinical parameters such as OGTT, triglycerides, VLDL, HDL-Cholesterol level (Bantle *et al*., 2004; Stanhope *et al*., 2009; Haung *et al*., 2011; Hudgins *et al*., 2011). Present study confirmed that high fructose diet led to reduction in glucose tolerance and increase in TG. High fructose consumption by rats has been shown to reduce glucose tolerance effects (Elliott *et al*., 2002; de Moura *et al*., 2009). High fructose diet, induce hypertriglyceridemia and other metabolic diseases (Basciano *et al*., 2005; Tappy and Kim, 2010). 32% fructose solution in diet of rats for 6 week led to 1.85 fold increase in TG (Robin *et al*., 1982). Rats fed with 66% of fructose in diet for two week increased the plasma TG levels increase 2 fold as compared to starch diet (Busserolles *et al*., 2002). Fructose consumption by rats has been shown to produce elevated TG, cholesterol, and body fat (Elliott *et al*., 2002; de Moura *et al*., 2009; Bocarsly *et al*., 2010).

Further research is needed to understand the regulation of microbiota during fructose diet and its relation to metabolic disease. Analysis of DGGE profile could be useful in understanding the dynamics of microbial population.

CHAPTER 5

Effect of probiotic E. coli 16 strain containing inulosucrase gene in alleviation of sucrose mediated metabolic disorder.

5.1 Introduction

Obesity has reached epidemic proportions globally. In 2007, it was estimated that more than 1.1 billion adults were overweight and at least 312 million of them clinically obese - and are major contributor to the global burden of chronic disease and disability (Hossain *et al*., 2007). Over the past 20 years, obesity rates have tripled in developing countries due to westernization of life style involving decreased physical activity and over consumption of cheap, energy-dense food products (Haslam and James, 2005). Overweight or obese pose a major risk for serious diet-related chronic diseases, including type 2 diabetes, cardiovascular disease, hypertension, and certain forms of metabolic disorders (Basciano *et al*., 2005). The health consequences range from increased risk of premature death, to serious chronic conditions due to high consumption of refined sugar such as fructose and sucrose (The term **'**Sugars' is used for 'total sugars' available in the diet, and includes all mono- and disaccharides present in foods and beverages, whether intrinsic (e.g. lactose from dairy products and sugars from fruit) or added sugars. Fructose, also known as laevulose, is found naturally in foods as a monosaccharide and as a component of plant oligosaccharides. Fructose is considerably sweeter than sucrose and its use enhances the flavours and physical appeal (e.g., colour stability, humectancy, and freezing point depression) of many foods and beverages. Because of its intense sweetness (**Table 5.1**), fructose has been used in place of sucrose and other carbohydrates to reduce overall carbohydrate and energy content of dietetic products (Havel *et al*., 2005; Tappy and Kim, 2010).

Table 5.1: Relative sweetness of sugars (White, 2008)

Relative Sweetness Scale - Sucrose = 100	
Compound	Rating
Sucrose	100
Fructose	140
High Fructose Corn Syrup	120-160
Glucose	70-80
Galactose	35
Maltose	30-50
Lactose	20

The consumption of fructose has increased greatly in the United States (**Fig. 5.1, Table 5. 2**), primarily as a result of increased use of high-fructose corn syrup in soft drinks and various confections and secondarily as a sucrose which is also used a sweetener in many food stuffs (Tappy and Kim, 2010). The increase in consumption of soft drinks and sugared fruit drinks is a critical factor in modifying the diet (Kantor *et al.*, 2008).

Fig. 5.1: Per capita consumption of calorific sweeteners (U.S. department of agriculture, Economic research service, 2010)

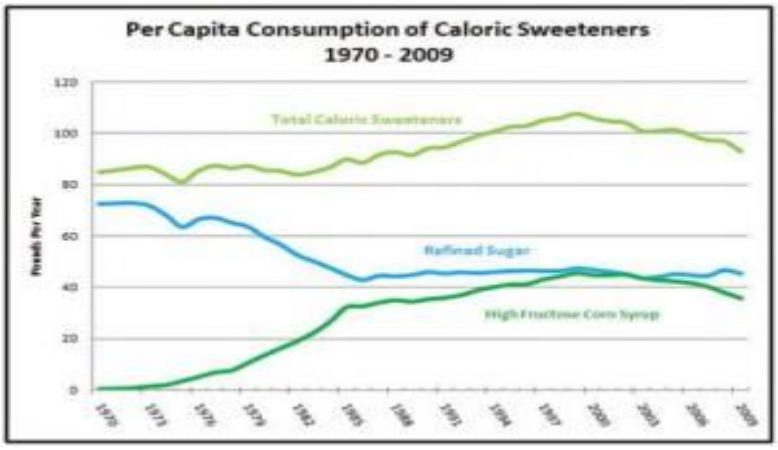
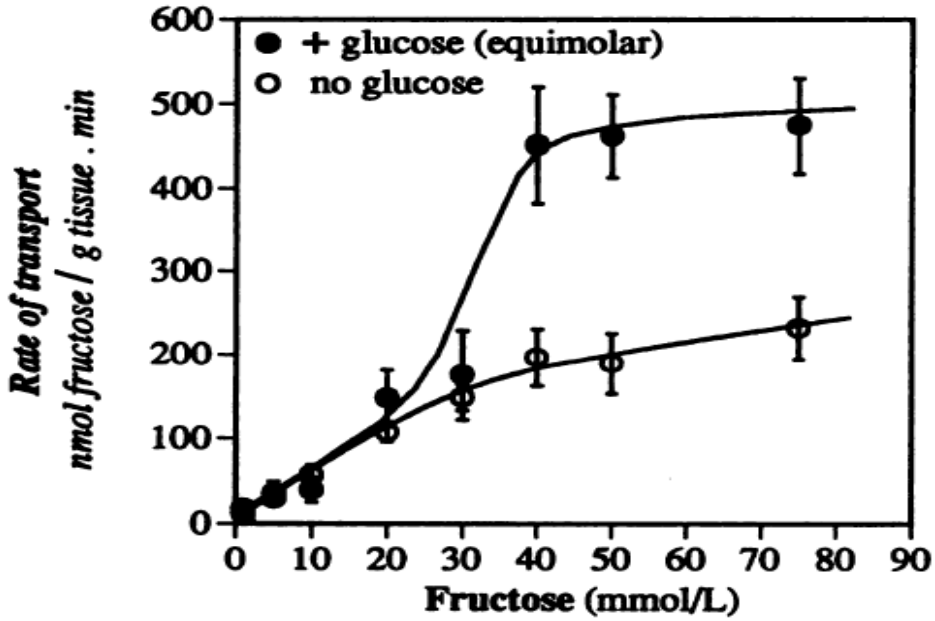


Table 5.2: World per capita consumption of sugar (Tappy and Kim, 2010)

	Per captia consumption of sugar, gm/day	
Continents	**1986**	**2006**
Europe	107	124
North America	83	88
South America	117	143
Asia	30	45
Africa	40	46
Oceanic	122	118

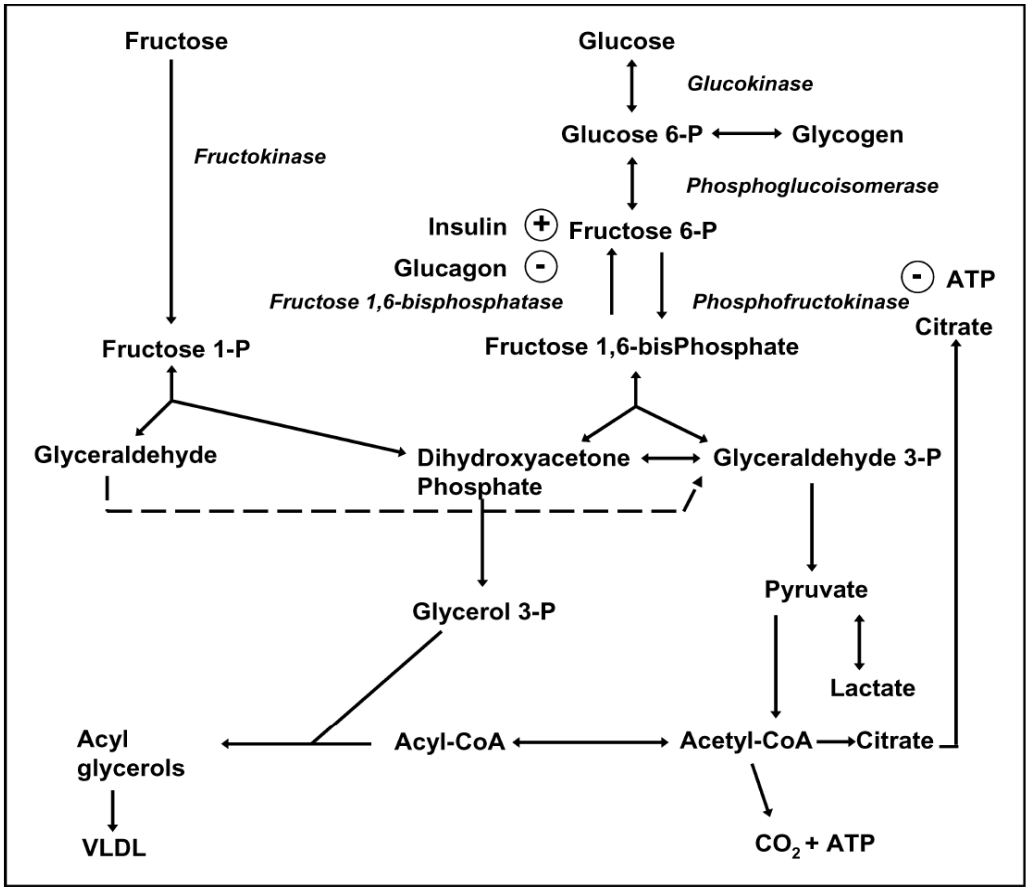
Fig. 5. 2: Fructose absorption in presence and absence of glucose (Berghe *et al*., 1986)



Fructose is readily absorbed and rapidly metabolized by human and rats liver (Berghe *et al*., 1986). Fructose absorption appears to be enhanced in the presence of glucose (**Fig. 5.2**). There is minimal utilization of fructose in peripheral tissues. Fructose can be phosphorylated at C6 position by hexokinase and at C1 position by fructokinase which occurs predominantly in the liver (**Fig. 5.3**). Glucose inhibits the phosphorylation of fructose by hexokinase thereby increasing the amount of unphosphorylated fructose in the diets of rats and humans which results in increases in the activity of fructokinase. Fructose-1-phosphate, which accumulates rapidly in the liver, is then cleaved to dihydroxyacetone phosphate and glyceraldehyde by fructose-1-phosphate aldoslase. Accumulation of fructose-1-phosphate causes depletion of ATP, inorganic phosphorus and increases degradation of nucleotides to uric acid (Berghe *et al*., 1986).

Metabolism of fructose occurred in the liver with the three fates of dihydroxyacetone phosphate **(Tappy and Kim, 2010; Fig. 5. 3)**. The two trioses formed by the cleavage of fructose-1-phosphate can follow three paths: (i) Dihydroxyacetone phosphate can be isomerized to glyceraldehyde phosphate and continue through the glycolytic pathway to ultimately yield pyruvate, which enters the citric acid cycle as acetyl coenzyme A under aerobic conditions. The acetyl coenzyme A can then be used as the substrate for fatty acid synthesis; (ii) Dihydroxyacetone phosphate can be reduced to glycerol-3-phosphate and provide the glycerol backbone for synthesis of triacylglycerols, phospholipids and other lipids; and (iii) Dihydroxyacetone phosphate can also be condensed with glyceraldehyde-3-phosphate by aldolase to form fructose-1,6-diphosphate, and ultimately to glucose or glycogen (the storage form of carbohydrate in the body).

Fig. 5.3: Pathway of fructose metabolism in liver (Tappy and Kim, 2010)



More fructose is converted to lipids than is glucose. Rats fed with 66% of their diet as fructose were more hypertriglyceridemic than rats fed 66% glucose or laboratory chow for 2 weeks. (Verschoor *et al.,* 1985). Incubation with 27.5 mM fructose resulted in incorporation of twice the amount of lipids as incubation with 27.5 mM glucose (Delhotal-Landes *et al* ., 1987). Thus fructose metabolism is highly lipogenic pathway with a greater propensity to increase serum triglycerides. Additionally, fructose is implicated in compromising human health in many different ways by its metabolism in the intestine and liver (Dekker, et al 2010). Thus fructose is the major cause of many dietary disorders in high and medium income countries (WHO, 2009). Strategy for sugar alternatives had limited success because of their side effects which led to intense efforts directed towards prebiotic products in alleviating the metabolic disorder caused by fructose (Kelly, 2008; 2009).

Prebiotics are defined as "nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or limited number of bacteria normally resident in the colon" (Gibson and Roberfroid *et al*., 1995). In order for a food ingredient to be classified as a prebiotic, it must; (i) Be neither hydrolysed nor absorbed in the upper part of the GI tract; (ii) Be a selective substrate for one or a limited number of beneficial bacteria commensal to the colon, which are stimulated to grow and/or are metabolically activated; (iii) Be able to alter the colonic flora in favour of a healthier composition; and (iv) Induce luminal or systemic effects that are beneficial to the host health. Among the food ingredients, nondigestible carbohydrates (oligo- and poly-saccharides), some peptides, proteins, and certain lipids (both ethers and esters) are candidate prebiotics. Because of their chemical structure, these compounds are not absorbed in the upper part of the GI tract and not hydrolysed by human digestive enzymes. Such ingredients could be called "colonic foods," i.e., foods entering the colon and serving as substrates for the endogenous colonic bacteria, thus indirectly providing the host with energy, metabolic substrates and essential micronutrients. Amongst the colonic foods, nondigestible carbohydrates are naturally occurring and are able to fulfil the criteria of prebiotics as defined above. Compounds such as resistant starch, non-starch polysaccharides (plant cell wall polysaccharides, hemicellulose, pectins and gums), and nondigestible oligosaccharides

are categorized as colonic food (Delzenne *et al*., 1994). However, even though they all can be classified as colonic foods, not all are prebiotics. Indeed for most of these substances, the process of colonic fermentation is rather nonspecific (**Table 5.3**). When ingested they stimulate the growth and/or metabolic activity of different bacterial species in the colon including species that are both potentially harmful and beneficial.

Table 5.3: Classification of certain carbohydrates as colonic food prebiotics (Delzenne *et al.*, 1994)

Carbohydrates	Colonic foods	Prebiotic
Resistant starch	Yes	No
Non starch polysaccharides		
Plant cell wall polysaccharides	Yes	No
Hemicelluloses	Yes	No
Pectins	Yes	No
Gums	Yes	No
Non digestiable oligosaccharides		
Fructooligosaccharides	Yes	Yes
Galactooligosaccharides	Yes	Yes
Soybean oligosaccharides	Yes	?
Glucooligosaccharides	?	No

Fructooligosaccharides (FOS) is the only prebiotic for which sufficient data had been generated to allow an evaluation of their possible classification as functional food ingredients (Roberfroid *et al*., 2007; Kelly, 2008; 2009). FOS is the common name for fructose oligomers that are mainly composed of 1-kestose (GFS2), 2-nystose (GF3) etc. in which fructose units are bound at β-2,1 position of sucrose by the transfructosylating enzymes like glucosyltransferase, fructosyltransferase and inulosucrase (Yun, 1996). The β-1 glycosidic bonds of FOS, including the first glucose-fructose bond, are not hydrolysed to a great extent by any mammalian digestive enzymes (Stone-Dorshow *et al*., 1987; Bach *et al*.,

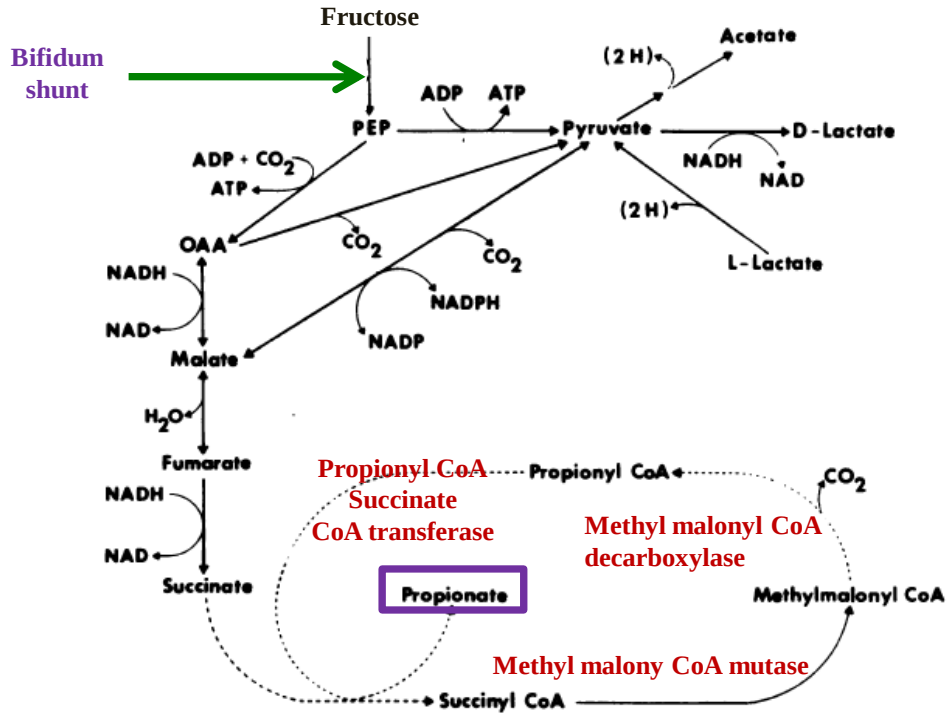
1995). An ileostomy subject has shown that ingested FOS is quantitatively recovered as nondigested material (Bach *et al*., 1995). Depending on the chain length, as defined by the number of fructosyl units and the degree of polymerization (DP), FOS are named oligofructose (DP <9, average DP = 4.8) or inulin (DP up to 60, average DP =12). Inulin is prepared by hot water extraction of chicory roots, and oligofructose is obtained by partial enzymatic hydrolysis of inulin under strictly controlled conditions. A number of common food stuffs such as garlic, onion, artichoke and asparagus have high oligofructose and inulin contents (Van loo *et al*., 1995). The term FOS will be used here to encompass both oligofructose and inulin.

FOS acts as a soluble fiber and can be used in increasing intestinal motility, transport and reduction of high levels of plasma cholesterol (Posada *et al*., 2002). FOS can decrease triacylglycerol and very low density lipoprotein (VLDL) when given to rats. The triacylglycerol lowering action is due to a reduction of *de novo* lipogenesis in the liver through inhibition of lipogenic enzymes (Wang *et al*., 1993). Post prandial insulin and glucose concentrations are low in serum of FOS fed animals and this could explain at least partially the metabolic effects of FOS. FOS feeding has resulted in antilipogenic effect in humans (Delzenne *et al*., 2001). Fermentation of FOS in colon leads to production of short chain fatty acids, mostly acetate, propionate and butyrate which are almost completely absorbed by the large intestine. *In vitro* experiments on comparative fermentation of inulin and FOS showed that both are rapidly and completely metabolized by the colonic bacteria into propionate and butyrate (Critteden, 1996). Butyrate is utilised by the enterocytes whereas propionate and acetate (produced in the gut) enter the liver via the portal vein. In hepatocytes, acetate is activated mainly by the cytosolic acetyl CoA synthetase II and then enters into cholesterogenesis and lipogenesis pathways (Sone *et al*., 2002). Conversely propionate is a competitive inhibitor of the protein controlling the transport of acetate into the liver cells (Delzenne *et al*., 2008), a phenomenon which contributes to decrease in lipogenesis and cholesterogenesis as observed in vitro in rat hepatocytes.

The production of high quantities of propionate through fermentation of FOS by *Bifidobacteria* has been attributed to explain the reduction in serum and hepatic cholesterol

and VLDL in rats fed with FOS (Delzenne *et al*., 2008). Feeding of FOS increased *Bifidobacterium* spp., SCFA concentrations, decrease *Fusobacterium* spp. and pH (O`Sullivan *et al*., 1996; Fuller *et al*., 1998). *Bifidobacterium* spp. selectively ferment FOS in preference to other carbohydrate sources like starch, fructose, pectin and polydextrose (Gibson *et al*., 1995). FOS also reduces the expression of genes coding Fatty Acyll Synthetase and Acetyl CoA carboxylase which are key enzymes in fatty acid synthesis in the liver but not in the adipose tissue of rats fed with a high fat/high sucrose diet (Kang *et al*., 2006). In addition to the lower *de novo* lipogenesis, prebiotics could also improve fatty acid oxidation via activation of hepatic peroxisomes proliferator-activator receptor (PPARα). However, PPARα KO (-/-) mice treated with FOS had no significant difference in hepatic and serum TG levels compared to controls (Cani *et al*., 2007). As mentioned earlier the 2, 1 ß-D fructan fructanhydrolase liberates fructose from FOS which in turn is converted to propionate through Bifidus pathway (**Fig. 5.4**).

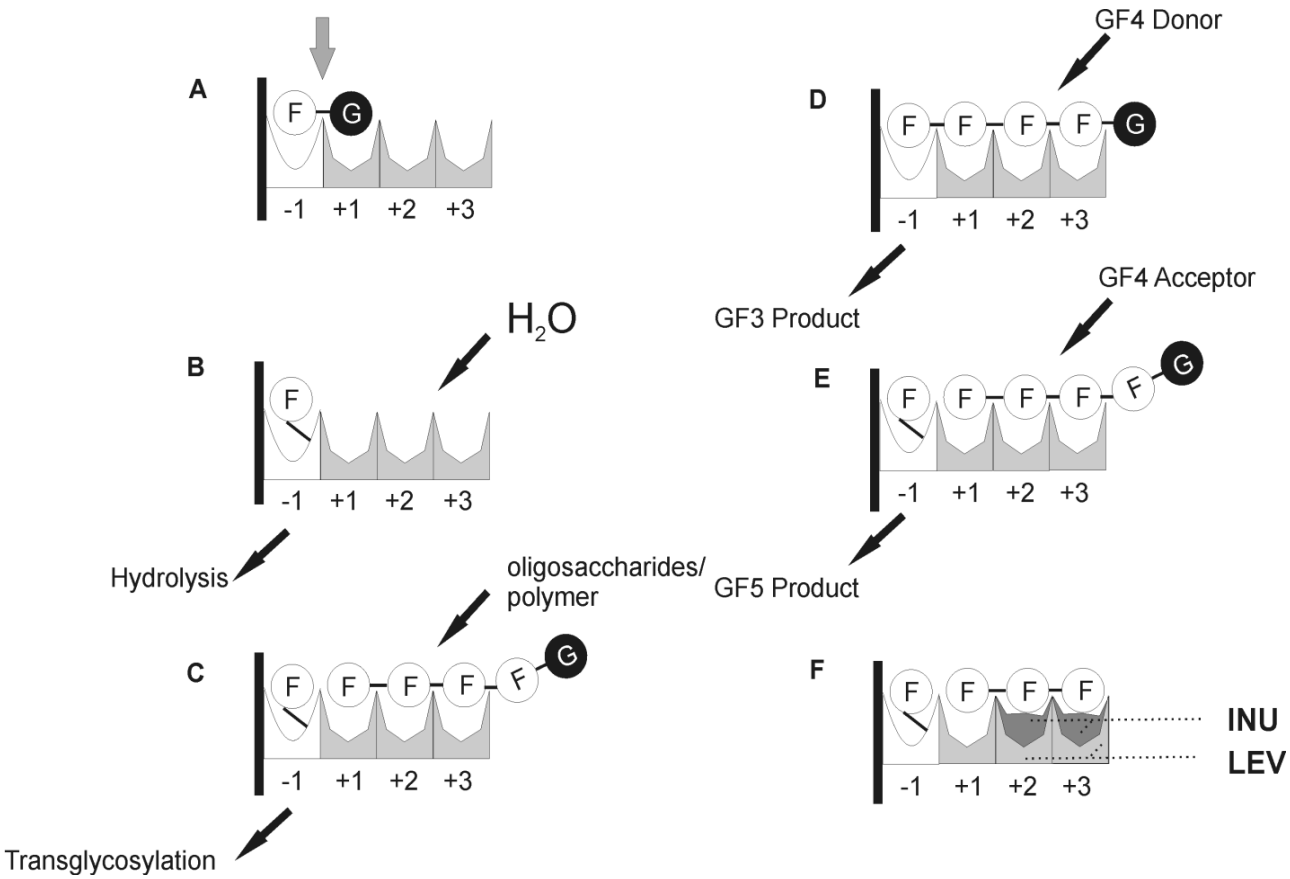
Fig. 5.4: Propionate production in *Bifidobacterium* spps.



Pathway for production of propionate

Naturally, oligosaccharides of fructose are produced by the polymerisation of fructose. Transfructolyating enzymes known as fructosyltransferases (FTF) present in plants and bacteria catalyze this process. Bacterial FTF enzymes belong to glycoside hydrolase family 68 (GH68) and catalyze synthesis of ß-linked glycosidic bonds with net retention of the anomeric configuration and / or hydrolysis. These FTF activities result in: (i) Trans glycosylation using sucrose, gluco- and FOS (oligosaccharide synthesis) or the growing fructan chain (polymerization) as acceptor substrates; (ii) Sucrose hydrolysis using water as acceptor. They are β-retaining enzymes employing a double displacement mechanism that involves formation and subsequent hydrolysis of a covalent glycosyl-enzyme intermediate (a Ping Pong type of mechanism). Most bacterial FTFs known are levansucrases (Lev; EC 2.4.1.10), synthesizing fructan polymers composed of β (2-6) linked fructose units (levan) and only limited information is available about bacterial inulosucrases (InuJ; EC 2.4.1.9) producing β (2-1) linked fructan polymers (inulin). Besides sucrose, raffinose (GalGF) but neither kestose (GF2) nor nystose (kestotetraose, GF3), can be used as substrates by the FTF enzymes of family GH68. However, FTFs can use various mono- and oligosaccharides (e.g. maltose, maltotriose, raffinose, arabinose, xylose, and sucrose) as fructosyl acceptor substrates in their transglycosylation reactions with sucrose as donor substrate (Dijkhuizen *et al*., 2002).

Fig. 5.5: Mechanism of FOS formation by inulosucrase (Dijkhuizen *et al*., 2002)



FTF enzymes is shown in **Fig. 5.5**. The donor and acceptor sub-sites of FTF enzymes are mapped out based on the available 3D structures. Binding of sucrose to subsites –1 and +1 (A) results in cleavage of the glycosidic bond, and formation of a (putative) covalent intermediate at sub-site -1 (B). Depending on the acceptor substrate used, hydrolysis (with water) or transglycosylation reactions may occur (with oligosaccharides or the growing polymer chain, resulting in FOS synthesis or polymer synthesis, respectively) (B and C).

Kestopentaose (GF4) is converted into GF3 and GF5 (D and E). The differences in affinity at the +2 and +3 sub-sites between *InuJ* and *Lev* is reflected by a shallow (dark grey) (low affinity) and deep cleft (light grey) (high affinity), respectively (F). Sugar binding sub-

sites are either shown in white (-1 sub-site) reflecting specific and constant affinity for binding of fructosyl residues only, or in light/dark grey (+1, +2, +3 sub-sites) reflecting their ability to bind either fructosyl, glucosyl with GFn substrate) or galactosyl (with raffinose) residues. The grey arrow indicates the position were cleavage/formation of glycosidic bond occurs. The black bar indicates the salt-bridge in FTF enzymes [E342 and R246 in SacB from *B. subtilis*] possibly blocking further donor sugar binding sub-sites.

This chapter deals with the efficacy of probiotic *E. coli* 16 expressing the *Lactobacillus johonsii* NCC533 *inuJ* gene encoding inulosucrase in alleviating the high sucrose related metabolic disorder.

5.2 Materials and Methods

5.2.1. Bacterial strains, plasmids and culture conditions

All strains and plasmids used in this study are listed in **Table 5.4**. The bacterial strains used in this study were *E. coli* 16 (Kumar *et al*., 2009), *E. coli* DH5α and *E. coli* BL-21. Strains of *E. coli* were grown at 37°C in LB medium. The minimal medium used had the following composition: 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 $MgSO_4$, thiamine and trace elements. Antibiotics were used at the following final concentrations: ampicillin 100 μg ml^{-1}. Plasmid pET15b-*inuJ* was a generous gift from Dr. Dijkhuizen. *E. coli* DH5α was used for constructing recombinant plasmids. *E. coli* BL21 was used for expressing the proteins, and for *in-vitro* and *in-vivo* experiments *E. coli* 16 was used. Plasmid-containing cells were grown in medium supplemented with 100 μg/ml ampicillin.

5.2.2. Construction of plasmids: pGRG-*gfp*, pGRG 8-16*gfp*, pMAL-p2Δlacl^{Q} and pMAL-p2Δlacl^{Q}-*inuJ*

gfp gene along with *lac* promoter (obtained as a PvuII fragment of 1.2kb) from pUC18-*gfp* plasmid was incorporated into SmaI site of pGRG36 plasmid to obtain pGRG-*gfp* plasmid. Similarly *vgb-gfp* with their promoter (obtained as a PvuII fragment of 3.1kb) from pUC 8-16-*gfp* plasmid was incorporated into SmaI site of pGRG36 plasmid to obtain pGRG 8-16-*gfp* plasmid. Temperature-sensitive plasmids pGRG-*gfp* and pGRG 8-16-*gfp* were maintained at 30°C. pMAL-p2 was digested with Mlu I/EcoRV, end filled and further self ligated, disrupting the *lacI*^{Q} gene to obtain constitutive pMAL-p2Δlacl^{Q} vector. Confirmation of disrupted clones of pMAL-p2Δlacl^{Q} was done on X-gal plate without IPTG. Plasmid pET15b-*inuJ*, containing inulosucrase gene, was digested with NcoI/BamHI to insert the 1.7 kb DNA fragment containing the gene into pMAL-p2Δlacl^{Q} for periplasmic expression. The recombinant plasmid was confirmed by restriction digestion.

5.2.3. Chromosomal integrations of *gfp* and *vgb-gfp* genes in *E. coli* 16

The chromosomal integration was done by using the Tn7 transposition mechanism (Craig *et al*., 2006). Electro-competent *E. coli* 16 cells were transformed by the plasmids pGRG-*gfp* and pGRG 8-16-*gfp* independently. Selected transformants were grown overnight in LB in presence of ampicillin at 30^0C. Grown culture was streaked on LA plate containing L-arabinose (0.1%) without ampicillin to induce *tns* genes, followed by incubation at 42^0C to cure plasmid. Further it was restreaked on LA plate to check the colony florescence and loss of ampicillin resistance.

Table 5.4. List of bacterial strains and plasmids used.

Plasmids/Strains	Relevant characteristics	References/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
pET15b- *inuJ*	Expression vector, derived from pET15b by insertion of a inulosucrase *inuj* gene; Ap^r	Dijkhuizen et al. 2008
pUC8:16-*gfp*	derived from the high-copy number vector pUC8:16 by insertion of a modified gfp gene; Ap^r	This study
pGRG-*gfp*	derived from low copy no. integration vector pGRG-36 by insertion of a modified *gfp* gene; Ap^r	This study
pGRG 8-16*gfp*	derived from low copy no. integration vector pGRG-36 by insertion of a *vgb* and modified *gfp* gene; Ap^r	This study
pMAL-p2Δ$lacI^Q$	deletion of $lacI^Q$ from periplasmic expression vector pMal-p2; Ap^r	This study
pMAL-p2Δ$lacI^Q$ -*inuJ*	derived from periplasmic expression vector pMAL-p2Δ$lacI^Q$ by insertion of *inuJ* ; Ap^r	This study
Bacterial strains		
E. coli BL21	*F′ ompT hsdSB (rB– mB–) gal dcm*	Sambrook and Russell. 2001
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 isolate16	Wild type	Prasant et al. 2009

5.2.4. Transformation of pMAL-p2Δlacl^{Q} and pMAL-p2Δlacl^{Q} –*inuJ* plasmid in *E. coli* 16 integrants.

The pMAL-p2Δlacl^{Q} and pMAL-p2Δlacl^{Q}–*inuJ* plasmids were independently transformed into *E. coli* 16-*gfp* chromosomal integrants using the $CaCl_2$ method. Similarly, plasmids pMAL-p2Δlacl^{Q} and pMAL-p2Δlacl^{Q} –*inuJ* were also independently transformed into *E. coli* 16-*vgb-gfp* chromosomal integrants using the $CaCl_2$ method. Transformants were selected on LA plates containing ampicillin 100µg ml^{-1}.

5.2.5. Preparation of *E. coli* cell extracts and *inuJ* activity assay.

E. coli BL-21, *E. coli* 16 and *E. coli* 16 integrants harboring pMAL-p2Δlacl^{Q} and pMAL-p2Δlacl^{Q} –*inuJ* constructs were grown overnight and harvested by centrifugation at 9,200 g for 2 min at 4°C. The cell pellet was washed twice 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 15 s in an ice bath, followed by centrifugation at 9,200 g at 4°C for 30 min to remove cell debris. The cell free extract was used for the inulosucrase assays. Periplasmic fraction (Kustu *et al.*, 1984) and extracellular fraction were also checked for the presence of the enzyme activity. Initial rate of the inulosucrase reaction was measured at 37°C in 50 mM potassium phosphate buffer (pH 7.0) in the presence of 500 mM sucrose. The enzyme inulosucrase catalyzes the formation of fructose polymers from sucrose in turn liberating glucose and thus this can be used as an indicator of the enzyme activity (Dijkhuizen *et al* ., 2006). Glucose was estimated using the DNSA method (Miller *et al*., 1959).

5.2.6. SDS-PAGE and Activity staining of *inuJ* gene

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) gel was performed as mentioned by Sambrook and Russell (2001) to detect inulosucrase polypeptide. Samples were mixed with an equal amount of 2X sample buffer (0.125 M Tris-HCl [pH 6.8], 1% SDS, 20% glycerol, and incubated at 90°C for 5 min, centrifuged at 10,000 rpm for 20 sec, and loaded onto a 12% slab gel. After electrophoresis, the gel was stained with Coomassie blue. Native polyacrylamide gel electrophoresis (PAGE)

gels were incubated at ambient temperature in McIllvaine's buffer (pH 7.0) with 10% sucrose. Formation of FOS within the gel led to white, turbid bands indicating the position of active enzyme. Prolonged incubation caused bursting of the gel due to excessive FOS formation at these sites (Hettwer *et al*., 1995).

5.2.7. Animal Experiments

5.2.7.1 Experimental animals:

Male Charles foster rats were housed in the Departmental animal house under controlled room temperature (21 ± 2 ºC). The experiments were carried out after the approval of Animal Ethical Committee of Department of Biochemistry, The M. S. University of Baroda, Vadodara, Gujarat, India (Approval No. 938/A/06/ CPCSEA), and CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) guidelines were followed.

5.2.7.2. *Invivo* localization of *E. coli* 16 integrants in Charles Foster rats

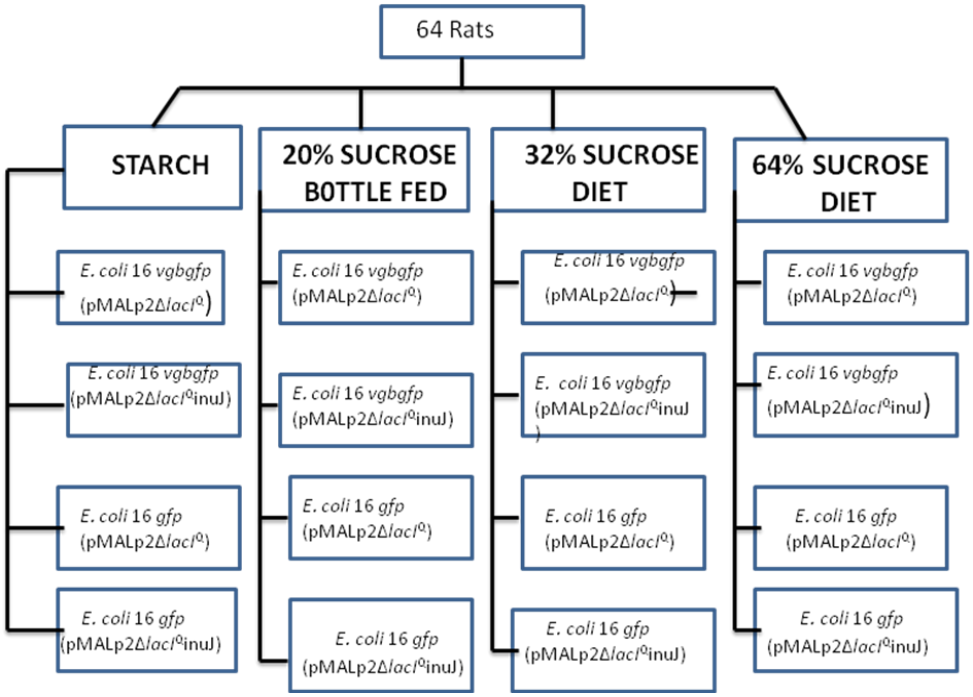
Charles foster rats of 2-3 months age, were given drinking water containing streptomycin sulfate (5 gml^{-1}) for 24 h to remove the existing resident facultative microflora and then starved for food and water for 18-20 h. Freshly grown culture (O.D. 0.8 to 1.2) was centrifuged at 5000rpm and then culture was resuspended in 0.85% saline and tube fed for 3 days and then one dose at weekly interval. After bacterial suspension was ingested, rats were given full access to food and water. *gfp* labeled bacteria was checked in the faecal sample. Fluorescence from transformed strains was detected using UV transilluminator at 365 nm.

5.2.7.3. Composition of standard and high-Sucrose diet (%).

The animals were divided into four groups of sixteen rats each. Group 1, control animals (Starch), received the control diet containing 64 percent starch and tap water *ad libitum* [64% starch, 20% casein, 0.7% methionine, 5% groundnut oil, 9.7% wheat bran and 3.5% salt mixture (The mineral mix in a kg contained $MgSO_4$ $7H_2O$., 30.5 g.; NaCl., 65.2 g.; KCl., 105.7 g.; KH_2PO_4., 200.2 g.; $MgCO_{3.,}$ 3.65g.; Mg $(OH)_2$. $3H_2O$., 38.8 g.; $FeC_6H_5O_7.5H_2O$., 40.0 g.; $CaCO_{3.,}$ 512.4 g.; KI.,0.8 g.; NaF., 0.9 g.; $CuSO_4.5H_2O$., 1.4 g.;

$MnSO_4$., 0.4 and $CONH_{3.,}$0.05 g.;)] and water *ad libitum*, 0.2 ml of vitamin mixture [The vitamin mix in a kg contained vitamin A concentrate I.P., 2500 I.U.; vitamin D3 cholecalciferol, 200 I.U.; thiamine hydrochloride, 0.5 mg; riboflavin, 0.5 mg; pyridoxin, 0.5 mg; sodium pantothenate, 1.5 mg; nicotinamide, 5 mg; ascorbic acid, 25 mg multivitamin tablets, Piramal healthcare Ltd., Mumbai, India] was added per kg feed. Group 2, (20% Sucrose bottle fed) 20% sucrose used as a bottle fed, received the diet similar in composition to the control diet of group 1 animals. Group 3 (32% Sucrose), Sucrose-fed animals received the sucrose enriched diet, which was similar in composition to the control diet (except starch and sucrose each 32%). Group 4 (64%Sucrose), sucrose-fed animals received the sucrose enriched diet, which was similar in composition to the control diet (except for starch which was replaced by 64% Sucrose). The diets were prepared fresh everyday based on the method of Cohen *et al* (1977) and the rats were divided into four diet groups. Each group contained four subgroups based on their Probiotic fed. Each sub group contained four rats each **Table 5.5**. All 64 rats were monitored with diet given every day and water provided *ad libitum*. On the 21 day, triglyceride level and feacal counts of probiotic were checked.

Table 5.5. Diet regime of all the rats. Each group contained 4 rats.



5.2.7.4. Short (28days) and Long (120 days) term effect of probiotic on 20% sucrose

Experiment was performed with 20% sucrose as bottle fed to groups mentioned in **Table 5.6.** Long term effects of probiotics were monitored in rats which were divided into 3 groups and treated at regular interval with genetically modified probiotic. Groups I: Rats were fed with chow diet Groups II: Rats were treated with 20% sucrose as bottle fed and Groups III: Rats were gave 20% sucrose as bottle fed with probiotic *E. coli* 16 gfp integrants harbouring inulosucrase plasmid at weekly interval.

Table 5.6: Short term effects (28 days) of probiotic on 20% sucrose received by animals

Group	Culture (fed at weekly interval)	Diet
Group I (Control)	-	Chow diet
Group II	*E. coli* 16	Chow diet
Group III (Positive control group)	-	Chow diet **with 20% sucrose**
Group IV	*E. coli* 16gfp	Chow diet **with 20% sucrose along with weekly doses of** *E. coli* 16gfp
Group V	*E. coli* 16vgb-gfp	Chow diet **witht 20% sucrose along with weekly doses of** *E. coli* 16vgb-gfp
Group VI	*E. c 16gfp* (pMALp2ΔlacIQinuj)	Chow diet **with 20% sucrose along with weekly doses of** *E. coli* 16vgb-gfp
Group VII	*E. c16vgb-gfp(*pMALp2ΔlacIQinuj)	Chow diet **with 20% sucrose along with weekly doses of** *E. coli* 16vgb-gfp

5.2.8. Biochemical parameters estimation.

5.2.8.1. Biochemical analyses in serum samples

SGOT, SGPT, biliburin, plasma glucose, total cholesterol, triglycerides, HDL and LDL cholesterol concentrations were estimated.

5.2.8.2. Determination of biochemical parameters in liver

A) Preparation of liver samples for biochemical estimation

Prior to biochemical analysis, each liver sample (100 mg/ml buffer) was homogenized in 50 mM phosphate buffer (pH 7.0); the homogenate was then centrifuged at 10,000 rpm for 15 min at 4^0C and the supernatant obtained was used for biochemical analysis. All liver parameters were expressed as activity per mg protein. The protein concentration in each fraction was determined by the method of Lowry (1951) using bovine serum albumin as a standard.

B) Determination of lipid peroxidation

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).

C) Quantitative analysis of enzyme activities

Catalase assay was measured by the method of Aebi *et al*. (1984) and Superoxide dismutase was determined by the method of Marklund and Marklund (1974), in which one unit was considered to be the amount of enzyme that inhibited pyrogallol autooxidation by 50%.

5.2.9. C**ytokine estimation in intestinal tissue.**

Full thickness strips of ileal segments weighing 0.08–0.12 g per strip were homogenized on ice in PBS [0.818 g of NaCl, 0.02 g of KCl, 0.02 g of KH_2PO_4 and 0.115 g of Na_2HPO_4 in 100 ml DDW (pH=7.2–7.4)] modified by adding 2.3376 g of NaCl, 0.5% of Bovine Serum Albumin, 0.05% of Tween 20 and protease inhibitor cocktail tablets for the complete inhibition of protease during tissue processing.

Homogenates were then centrifuged at 4 °C for 1 h at a speed of 11,000 rpm, and samples were taken from the supernatant (Barada *et al*., 2006) and used for cytokine estimation. For estimation cytokine multiplex bead array (Rat Millipore Kit, Millipore France) was used to quantitatively estimate cytokine levels in tissue from the controls and sucrose fed group rats. The assay was performed according to the manufacturer's instructions and data was acquired using a Bioplex system (Biorad, USA) as per Millipore multiplex kit setting. The cytokine analysis was performed using Bioplex array software (Biorad, USA).

5.2.10. Statistical analysis

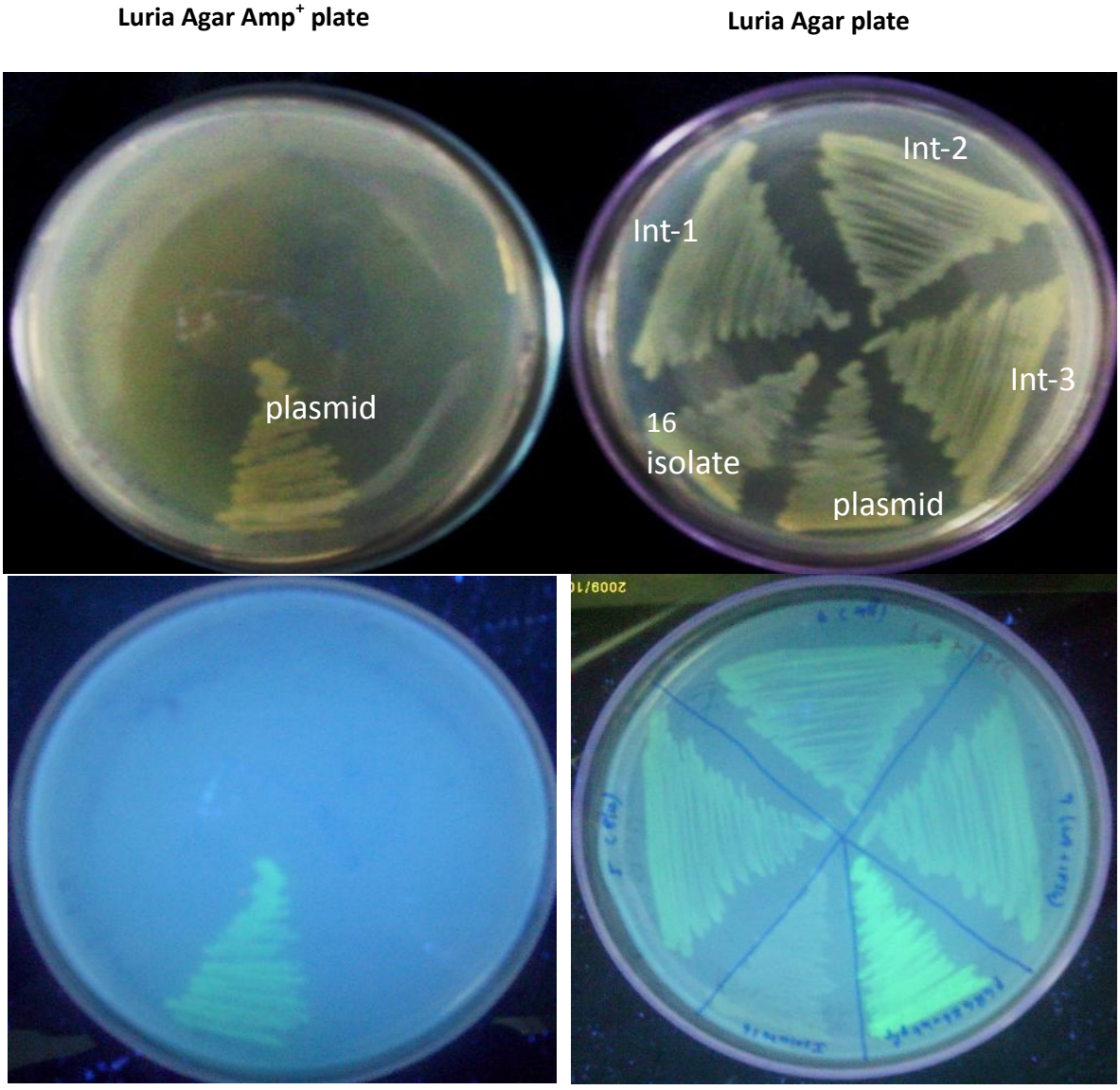
Statistical evaluation of the data was done by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test and results were expressed as mean ± SEM using Graph Pad Prism version 3.0 for Windows, Graph Pad Software,San Diego, California, USA.

5.3. Results

5.3.1. Characterization of integrants of *gfp* and *vgb-gfp* in probiotic *E. coli* 16.

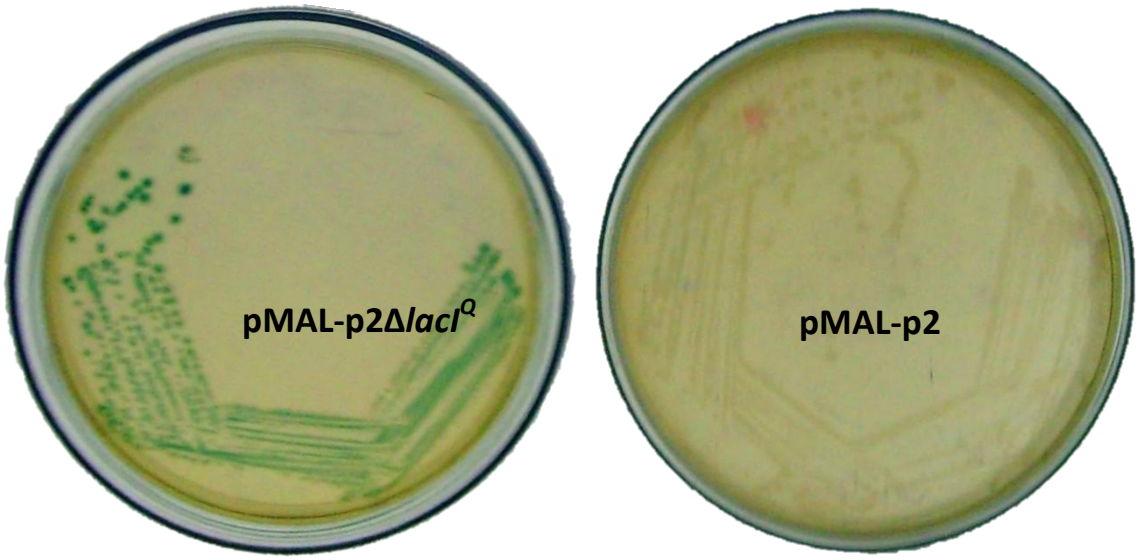
The *E. coli* 16 integrant (pGRG-*gfp* and pGRG *vgb-gfp*) showed loss of the antibiotic resistance and lesser fluorescence compared to the plasmid transformants which was accounted by the fact that the integrant single copy number (**Fig. 5.6**).

Fig. 5.6: Ampicillin sensitivity and green fluorescence of integrant of *E. coli* 16 strain



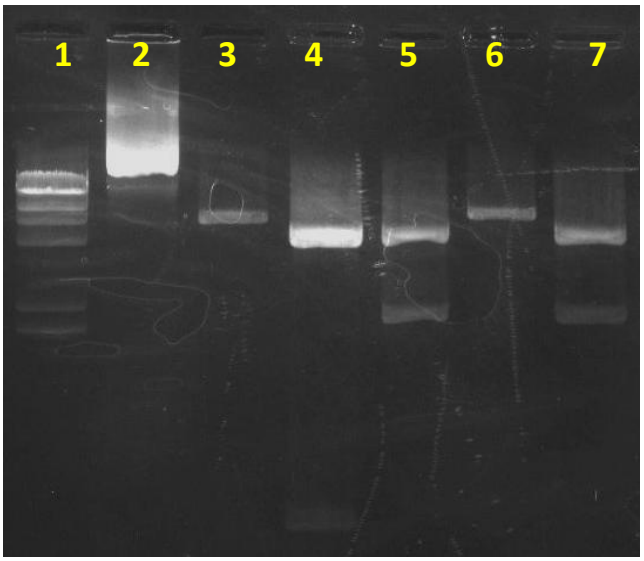
E. coli 16 integrants harboring pMAL-p2Δ*lacI*^{Q} plasmid showed constitutive phenotype on X-gal plate due to disruption of lacI^{Q} repressor binding protein site **(Fig. 5.7)**.

Fig. 5.7: Phenotypic confirmation of pMAL-p2Δ*lacI*^{Q} clones.



Insertion of inulosucrase gene in pMAL-p2Δ*lacI*^{Q} was confirmed by release of 2.3 kb fragment upon digestion with SalI and BglII enzymes (**Fig. 5.8)**.

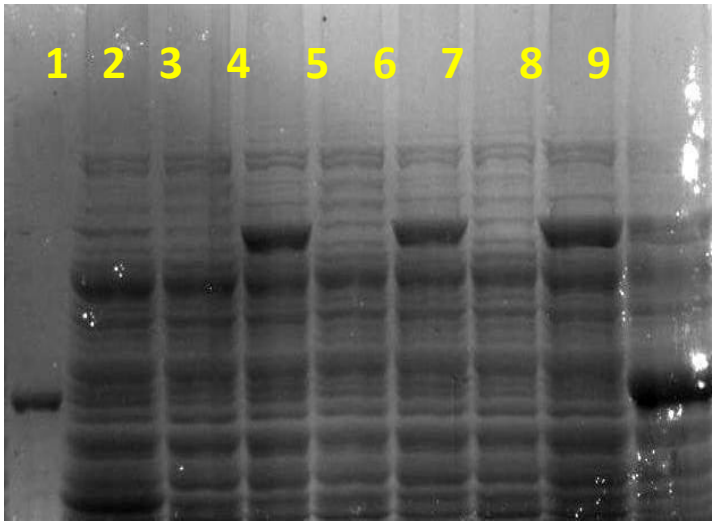
Fig. 5.8: Restriction digestion pattern for pMAL-p2ΔlacI^{Q}-*inuJ* plasmids



Lane1: - ƛ HindIII Marker
Lane2: - Plasmid of pMAL-p2ΔlaclQ-*inuJ*
Lane3: - pET-*inuJ* 7.3kb
Lane4:- pMAL-p2ΔlaclQ 5.4kb and 0.8kb.
Lane5:- pMAL-p2ΔlaclQ-*inuJ* 5.4kb and 2.3kb.
Lane6:- pET-*inuJ* 7.3kb
Lane7:- pMAL-p2ΔlaclQ-*inuJ* 5.4kb and 2.3kb

SDS PAGE of cell free extract of *E. coli* BL21 (DE3) containing InuJ showed the presence of 63 kD protein but in the clones a band was seen at 106 kD region. This is because InuJ protein is obtained as MBP-InuJ as a translational fusion protein with a molecular weight of 106 kD **(Fig. 5. 9).**

Fig. 5.9: SDS-PAGE analysis of the *E. coli* BL21 transformants containing the recombinant pMAL-p2ΔlacI^{Q}-*inuJ* plasmids

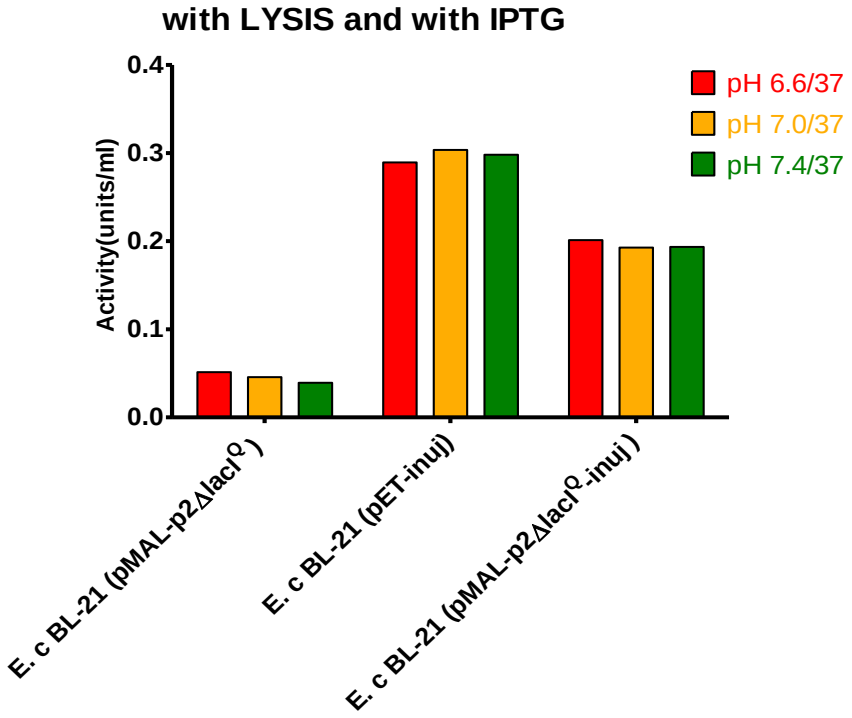


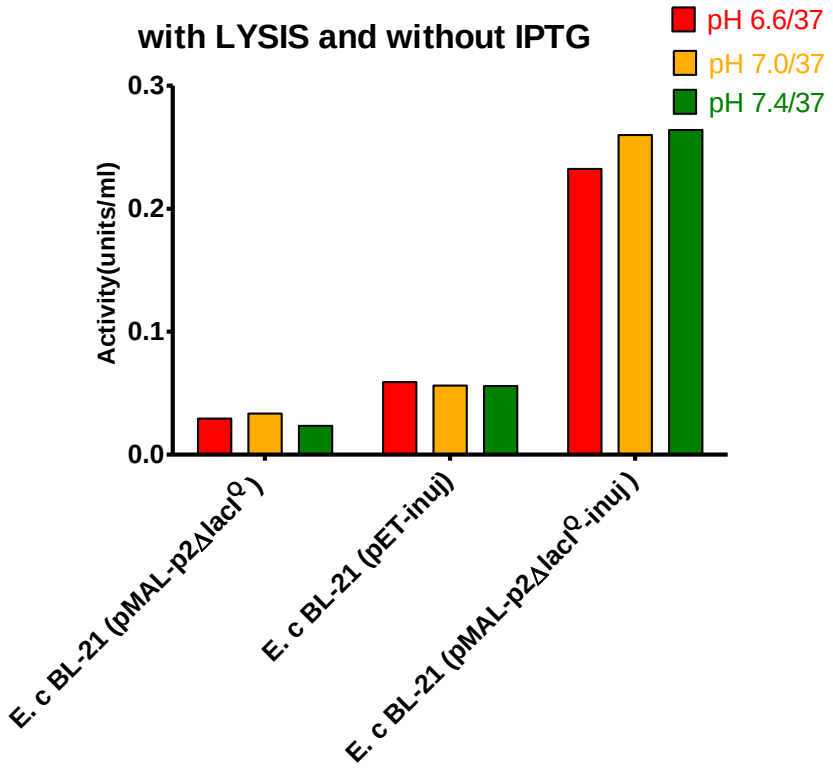
Lane 1 BSA 66kd
Lane 2 pMAL-p2 control
Lane 3 pMAL-p2ΔlacI^{Q}
Lane 4 pMAL-p2ΔlacI^{Q}-*inuJ* 106kd
Lane 5:- pMAL-p2ΔlacI^{Q}
Lane 6 pMAL-p2ΔlacI^{Q}-*inuJ* 106kd
Lane 7 pMAL-p2ΔlacI^{Q}
Lane 8 pMAL-p2ΔlacI^{Q}-*inuJ* 106kd
Lane 9 pET-*inuJ* 63kd

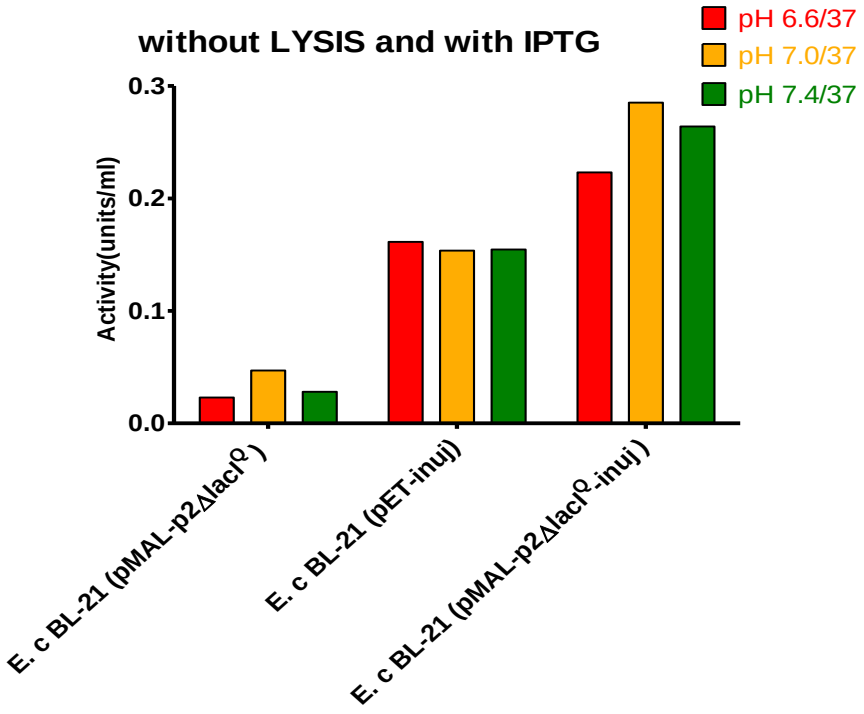
5.3.2 Constitutive expression of preiplasmic expression pMAL-p2ΔlacI^{Q}-*inuJ*.

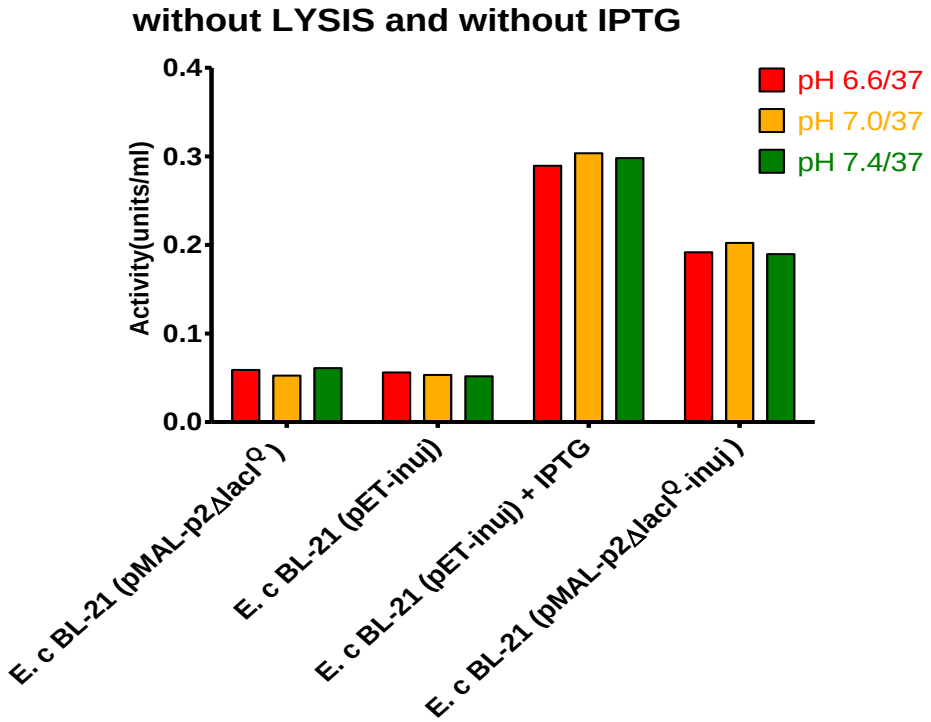
Constitutive periplasmic expression of pMAL-p2ΔlacI^{Q}-*inuJ* of the transformants was determined by monitoring the activities of four sets at three different pH**.** Activity was seen in all *E. coli* BL21 transformants except control. Activity was observed in the pellet in the absence of IPTG whereas the pET-*inuJ* control showed significant increase in activity upon addition of IPTG. Thus the cloned gene was producing a functional protein in *E. coli* BL21 (DE3). Inulosucrase activity without IPTG induction in pMAL-p2ΔlacI^{Q}-*inuJ* plasmid clearly demonstrated the disruption of *Iac*I^{Q} site (**Fig. 5.10**).The plasmid pMAL-p2ΔlacI^{Q}-*inuJ* was transformed into *E. coli* 16 integrant of *gfp* and *vgb-gfp* and then the enzyme assay was performed

Fig. 5.10 Optimization of inulosucrase activity in different condition





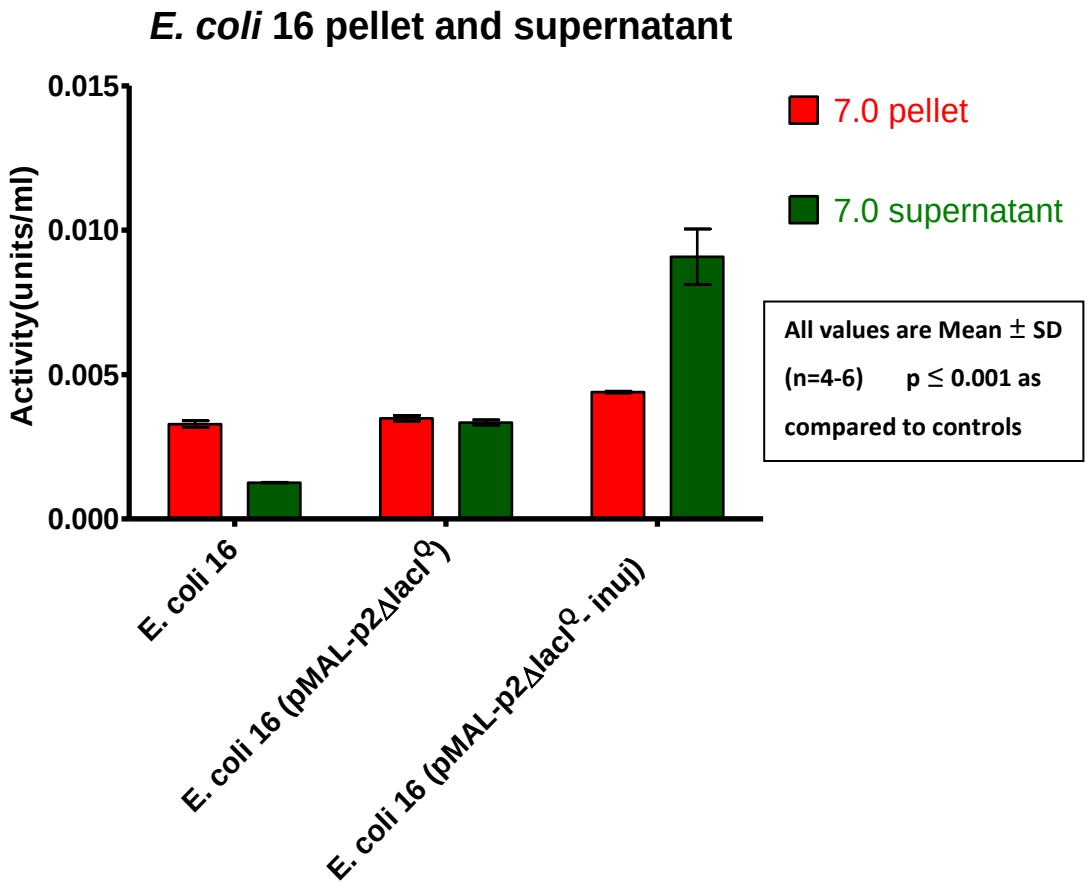

without LYSIS and with IPTG
pH 6.6/37
pH 7.0/37
pH 7.4/37
Activity(units/ml)
0.3
0.2
0.1
0.0
E. c BL-21 (pMAL-p2ΔlacIQ)
E. c BL-21 (pET-inuj)
E. c BL-21 (pMAL-p2ΔlacIQ-inuj)

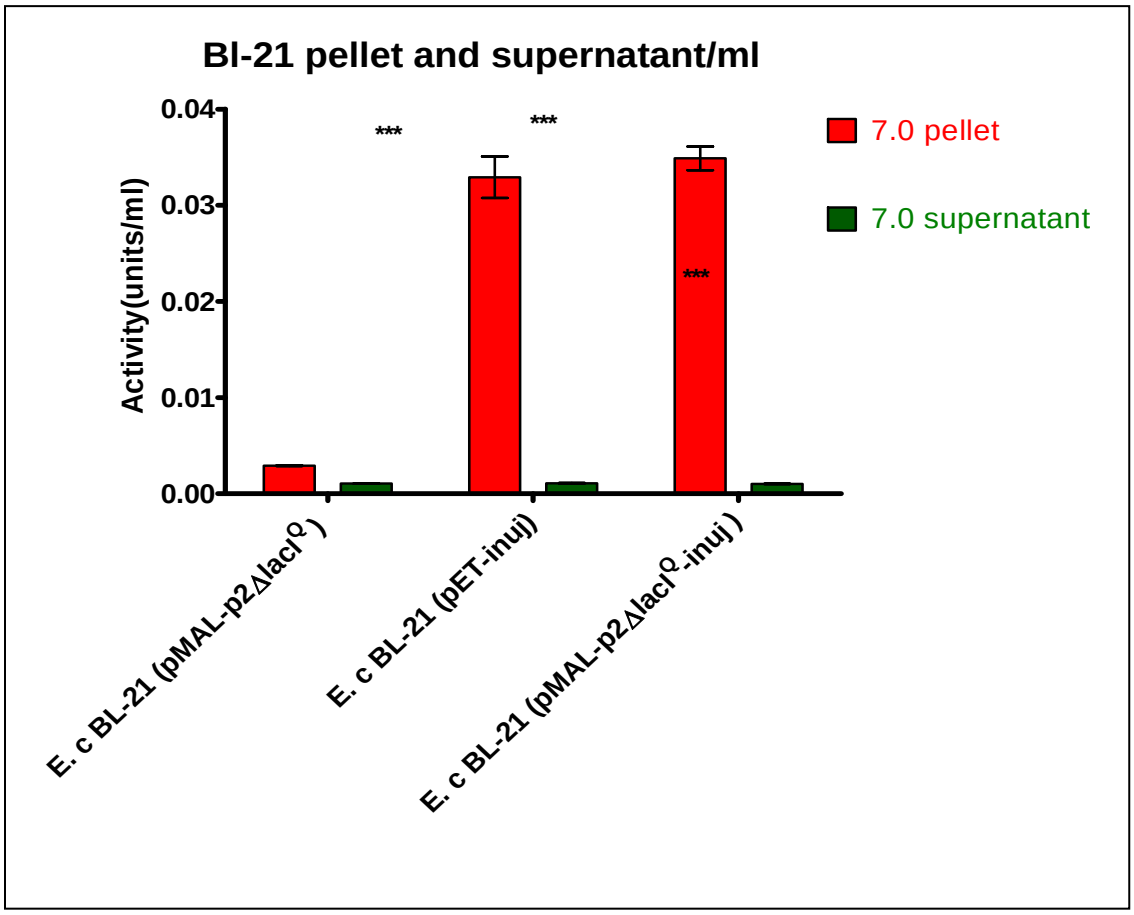

without LYSIS and without IPTG
pH 6.6/37
pH 7.0/37
pH 7.4/37
Activity(units/ml)
0.4
0.3
0.2
0.1
0.0
E. c BL-21 (pMAL-p2ΔlacIQ)
E. c BL-21 (pET-inuj)
E. c BL-21 (pET-inuj) + IPTG
E. c BL-21 (pMAL-p2ΔlacIQ-inuj)

5.3.3 Inulosucrase activity in the supernatant of *E. coli* 16 integrants containing pMAL-p2ΔlacI^{Q}-*inuJ* plasmid

The enzyme activity in *E. coli* 16 integrants (pMAL-p2ΔlacI^{Q}-*inuJ*) was performed in the supernatant along with pellet. *E. coli* 16 integrants (pMAL-p2ΔlacI^{Q}-*inuJ*) showed significant increase in activity in the supernatant than the lysate (pellet) as compared to the controls. Thus, enzyme activity was also carried out in *E. coli* BL21 (pMAL-p2ΔlacI^{Q}-*inuJ*) to determine its secretion into the medium. Interestingly, the supernatant of *E. coli* BL21 (pMAL-p2ΔlacI^{Q}-*inuJ*) did not show any activity but showed activity in the lysate **Fig. 5.11**.

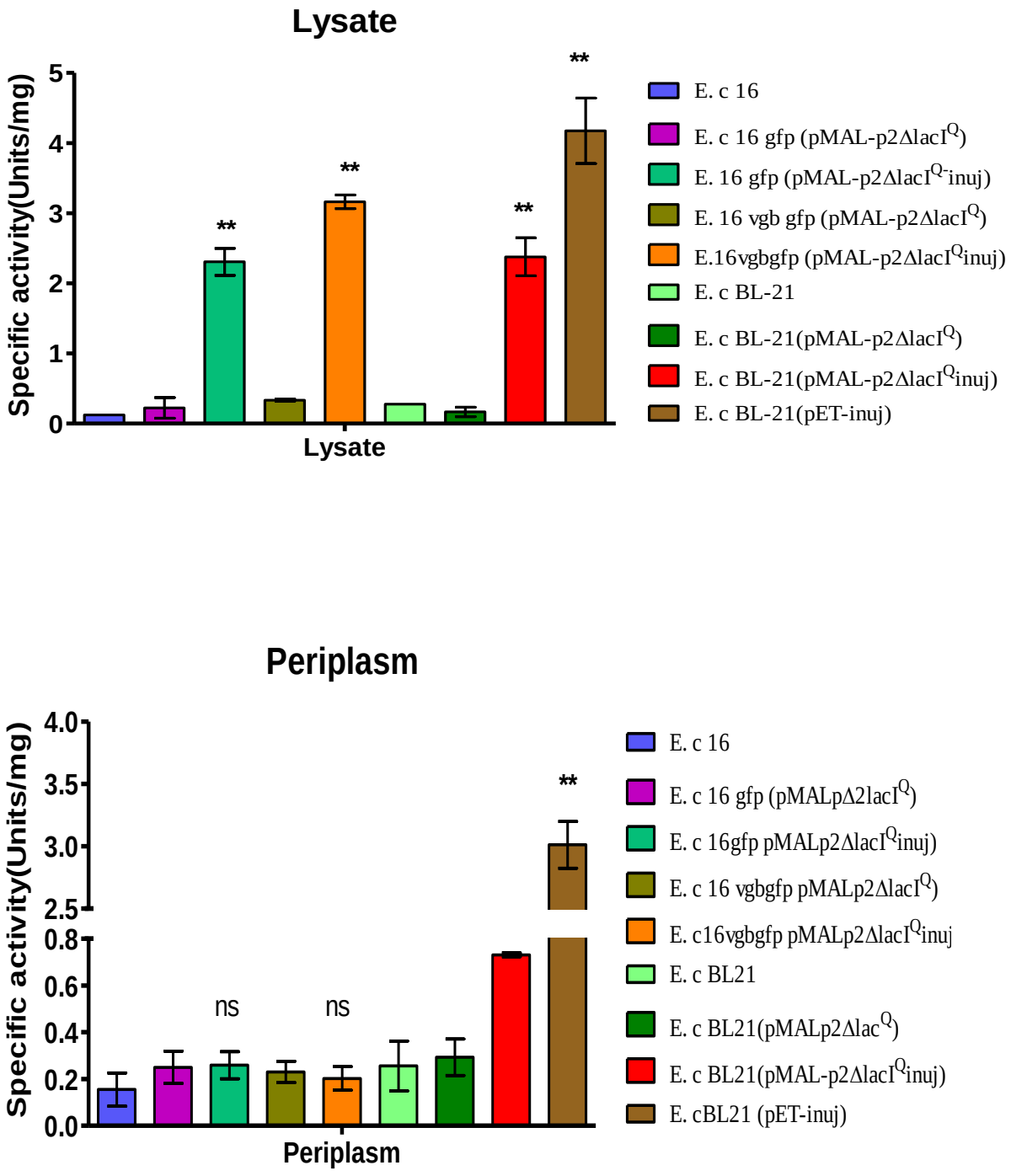
Fig. 5. 11: Activity of inulosucrase enzyme in *E. coli 16* integrant and BL21 containing pMAL-p2ΔlacI^{Q}-*inuJ*

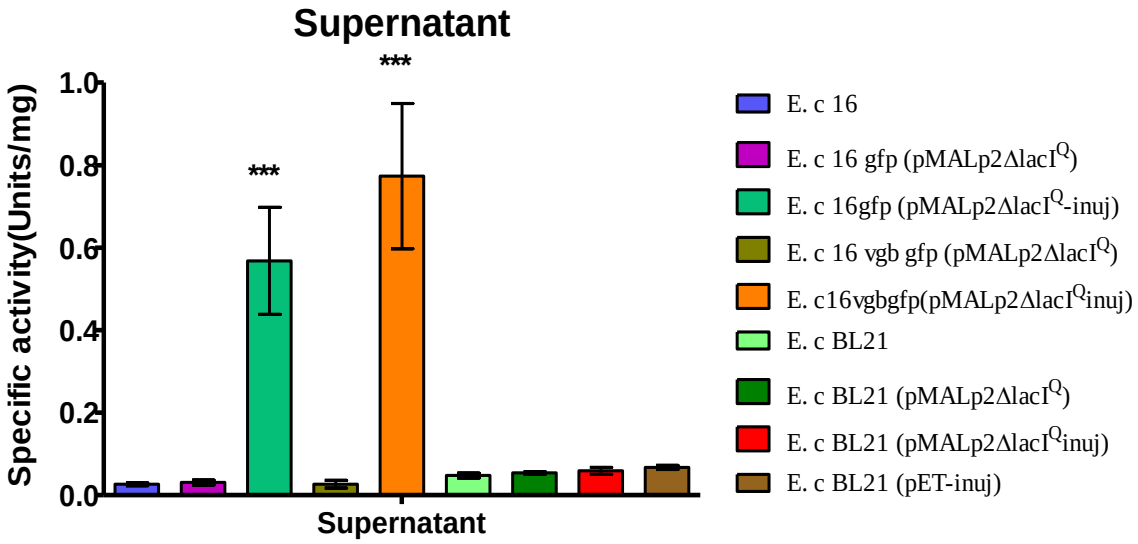




Thus, only the *E. coli* 16 integrants (pMAL-p2ΔlacI^{Q}-*inuJ*) transformants showed the protein in the supernatant. Another set of experiment was carried out to check for the presence of the enzyme in the periplasm. Periplasmic proteins were extracted by using the method of Kustu *et al*., (1984). Activities in all three fractions of protein-total (lysate), periplasmic and supernatant were determined (**Fig 5. 12**).

Fig. 5. 12: Specific activity of inulosucrase enzyme in supernatant, periplasm and lysate of *E. coli* 16 integrants and BL-21 strain containing (pMAL-p2ΔlacI^{Q}-*inuJ*).

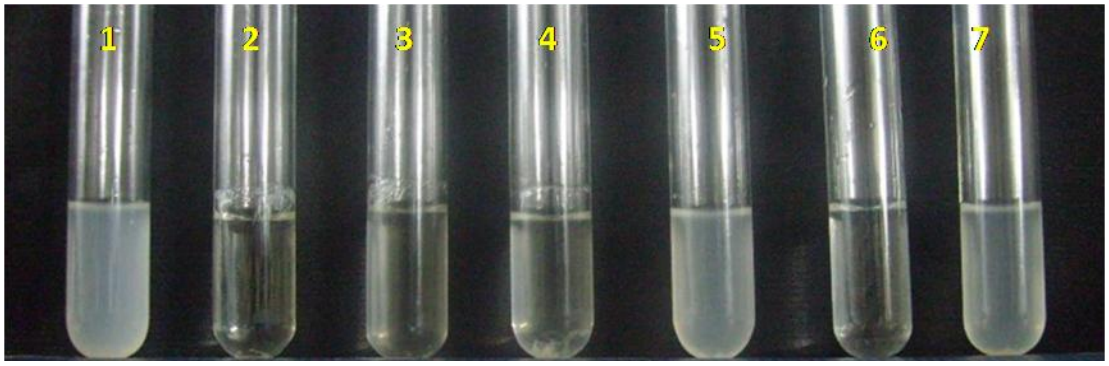




All values are Mean ± SD (n=4-6) $p \leq 0.001$ as compared to controls

In case of *E. coli* BL21 transformants, both the lysate and the periplasmic fractions contained the activity while in case of the transformants of *E. coli* 16 only the supernatant showed appreciable activity. The transformants were checked for their ability to grow on sucrose. Normally *E .coli* does not grow on sucrose but the transformant containing InuJ enzyme coverts sucrose to give FOS and simultaneously releases glucose which can be utilised for growth **(Fig. 5.13).**

Fig. 5. 13: Growth of inulosucrase transformants in presence of sucrose.



1.*E. coli* BL21 (DE3) containing pET-15b-*inuJ* with IPTG; 2. *E. coli* BL21 (DE3) containing pET-15b-*inuJ* without IPTG; 3. *E. coli* BL21; 4. *E. coli* 16 containing pMAL-p2Δ*lacI*^{Q}; 5.

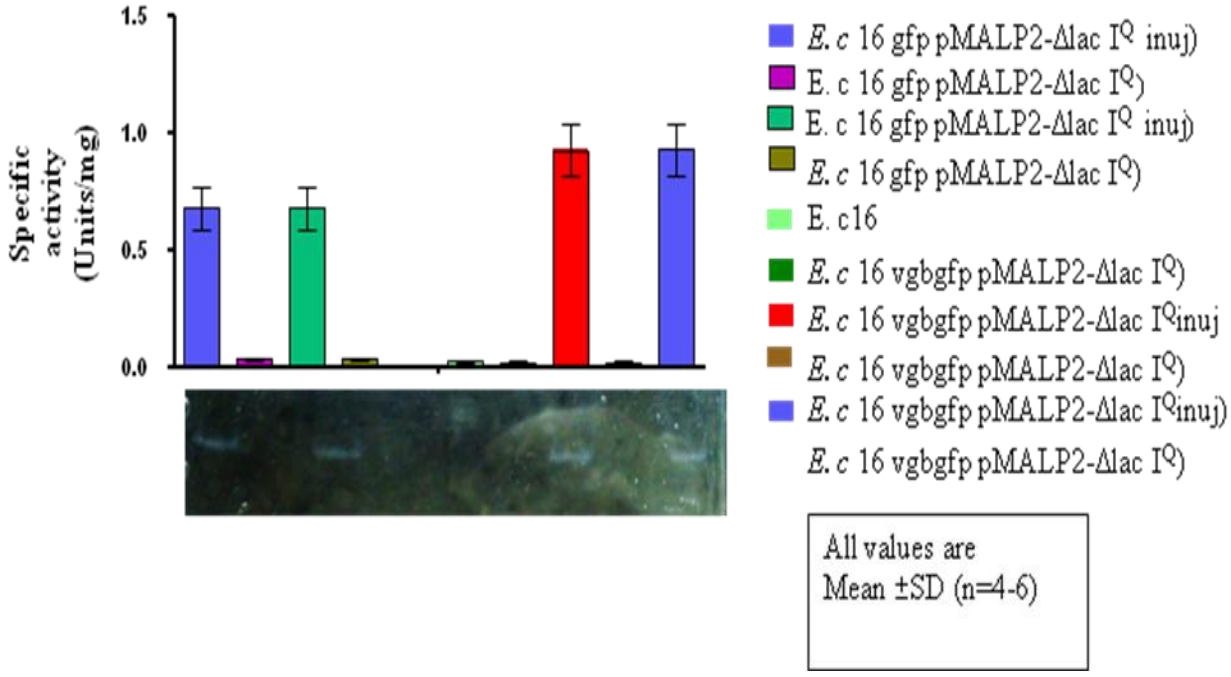
E. coli 16 containing pMAL-p2Δ*lacI*^{Q} *inuJ;* 6. *E. coli* 16; 7. *E. coli* 16 integrant containing pMAL-p2Δ*lacI*^{Q} *inuJ*.

The clones containing the functional enzyme indeed grew on sucrose while the vector controls and wild types did not grow in the presence of sucrose.

5.3.4 Activity staining of inulosucrase.

InuJ activity staining was performed to confirm the generation of fructose oligosaccharides polymer (FOS) (Rudolph *et al*., 1995). *E. coli* 16 (pMAL-p2Δ*lacI*^{Q} *inuJ*) showed the formation of the polymer (**Fig. 5. 14**).

Fig. 5. 14: Activity staining of E. c 16 harboring inulosucrase



5.3.5 Determining the effectiveness of *E. coli* 16 integrant harboring *inuJ* gene on sucrose fed rats

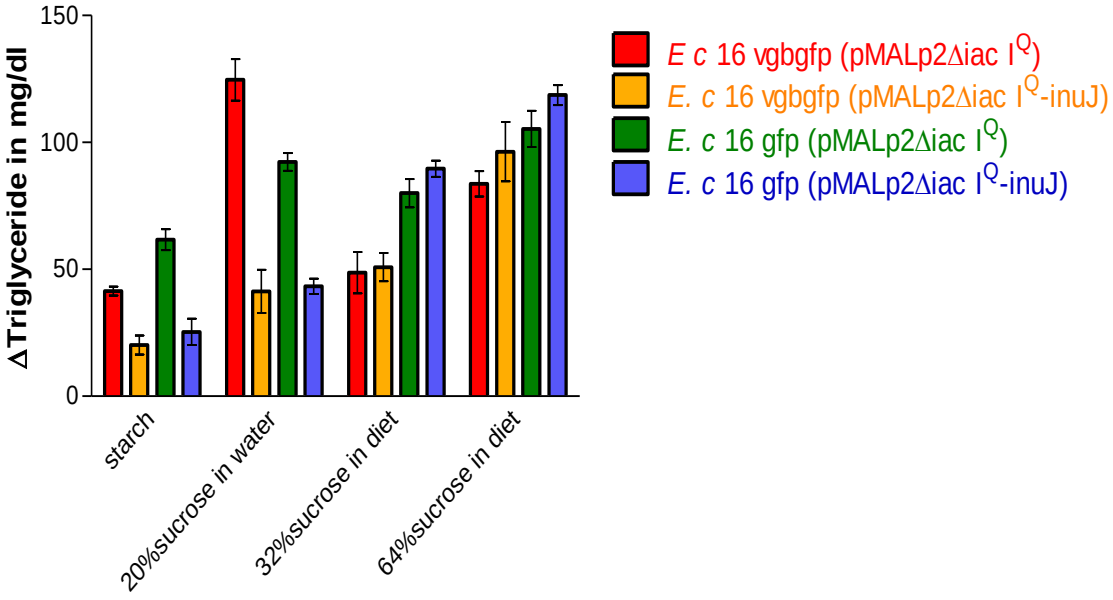
Change in triglycerides (TG) levels in serum samples were checked at the start of experiment and after 21 days of starch and sucrose fed diet in rats (**Fig. 5.15**). The change in

TG level were significant (P<0.01value) in 20% sucrose fed groups. In feacal sample, *E. coli* 16 integrant colonies showed fluorescence, antimicrobial activity and ampicillin resistance. After 21 days, approximately 10^{5-7} colonies of *E. coli* 16 integrants were present in per gram of feacal samples in all groups.

Table 5.7. Bacterial isolates count in feacal samples of rats.

	***E. coli* 16vgbgfp pMALp2ΔlacI^{Q}**	***E. coli* 16vgbgfp pMALp2ΔlacI^{Q}-inuj**	***E. coli* 16gfp pMALp2ΔlacI^{Q}**	***E. coli* 16gfp pMALp2ΔlacI^{Q}- in**
Starch	4.50 X 10	1.15 X 10^{7}	8.90 X 10^{7}	4.40 X 10^{6}
20%Sucros	3.72 X 10	9.10 X 10^{7}	6.60 X 10^{7}	4.95 X 10^{6}
32%Sucros	6.50 X 10	2.85 X 10^{6}	8.50 X 10^{6}	1.25 X 10^{5}
64%Sucros	1.20 X 10	1.07 X 10^{7}	5.25 X 10^{7}	3.40 X 10^{6}

Fig. 5. 15. Change in TG level after 21days.



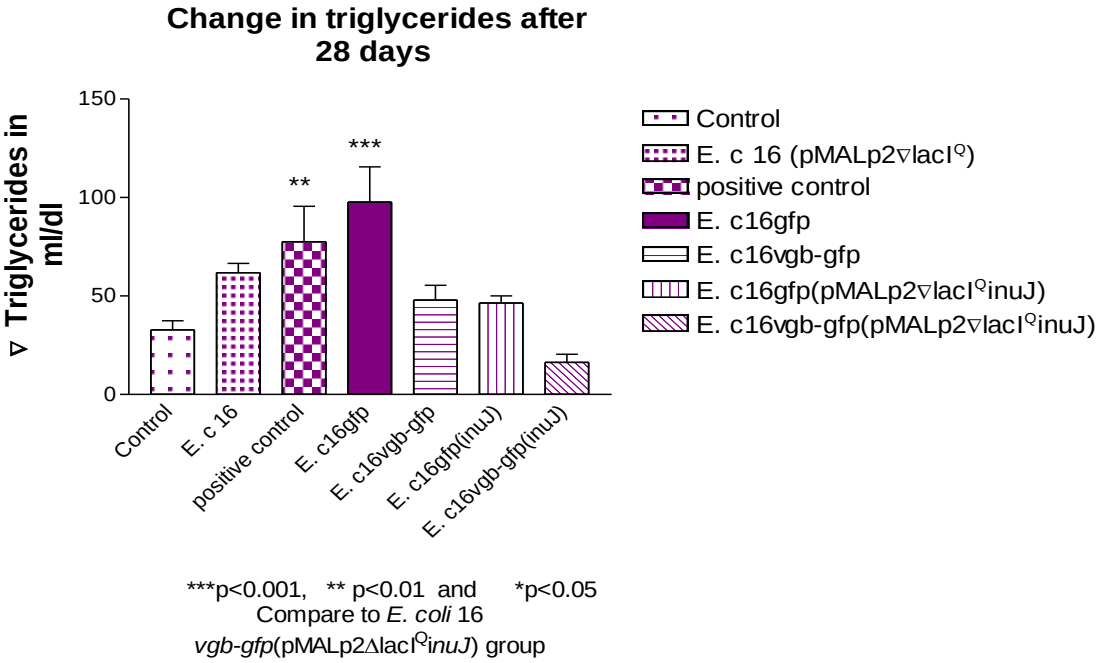
In the group of rats fed with 20% sucrose, the rats fed with *E. coli* 16 integrants containing *inuJ* plasmid showed a marked decrease in the TG level after 21 days as compared to the vector controls group. The other diet groups did not show much difference.

5.3.6. Short term effects (28 days) of probiotic harbouring *inuJ* gene.

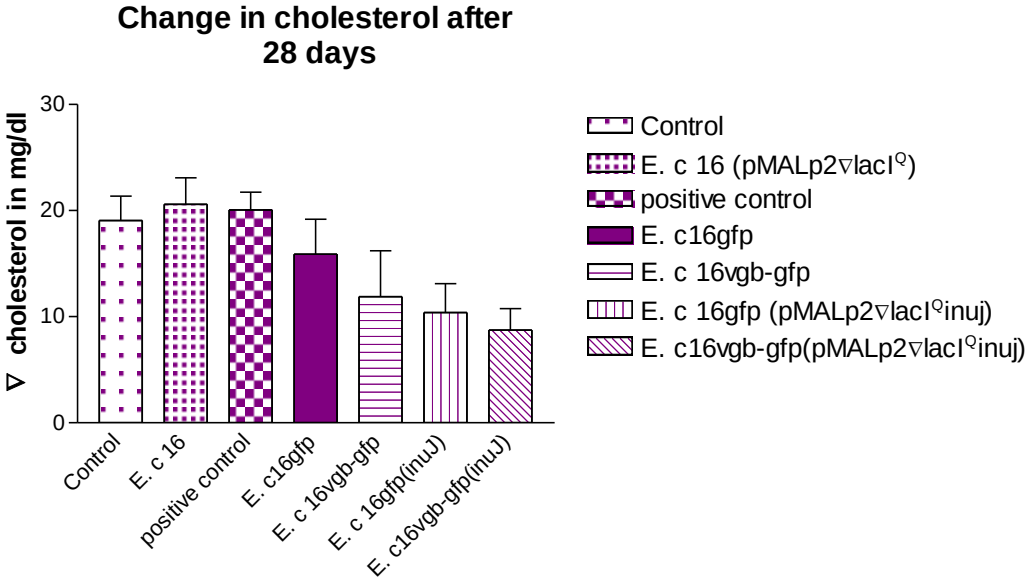
5.3.6.1. Serum Lipid profile.

Change in TG after 28 days were significantly increased ($p < 0.01$) in 20% sucrose fed positive control group compared to 20% sucrose fed along with InuJ group (*E.c*16vgb-gfp (pMALp2Δ*lacI*^{Q}*inuJ*)) **Fig.5.16** . No Significant change was observed in TG of 20% sucrose fed positive control groups and *E. c*16gfp group. Also no significant change was found in cholesterol levels between all groups. However, HDL cholesterol levels in InuJ fed rats were near to control group.

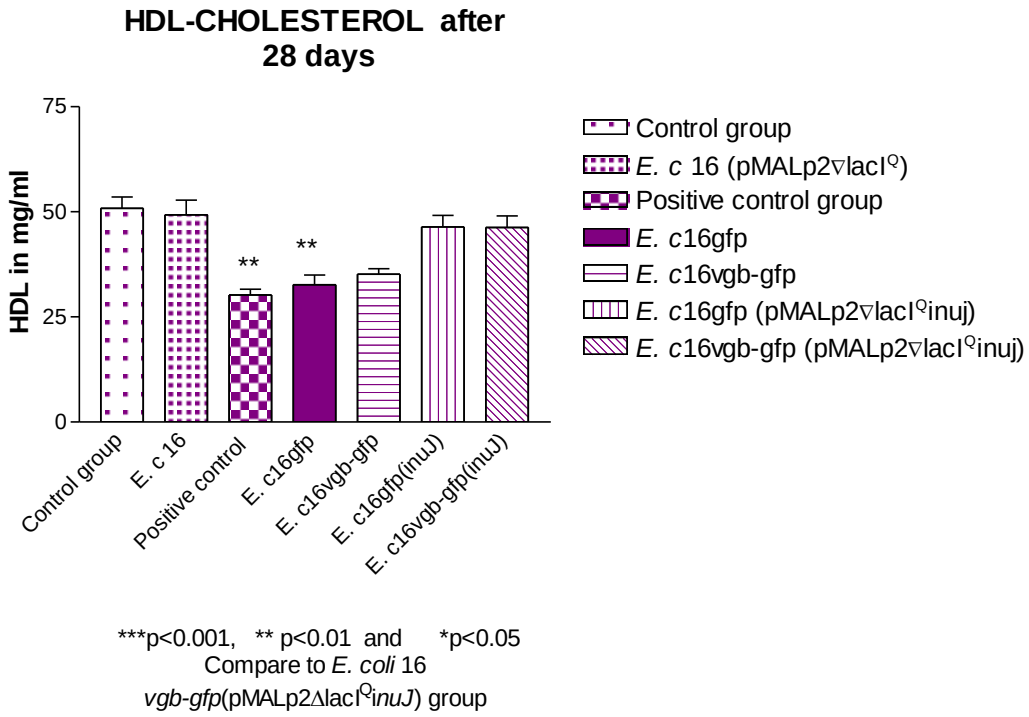
Fig. 5.16. Serum lipid profiles in rats fed sucrose diets for 28 days



***p<0.001, ** p<0.01 and *p<0.05
Compare to *E. coli* 16
vgb-gfp(pMALp2ΔlacI^{Q}*inuJ*) group



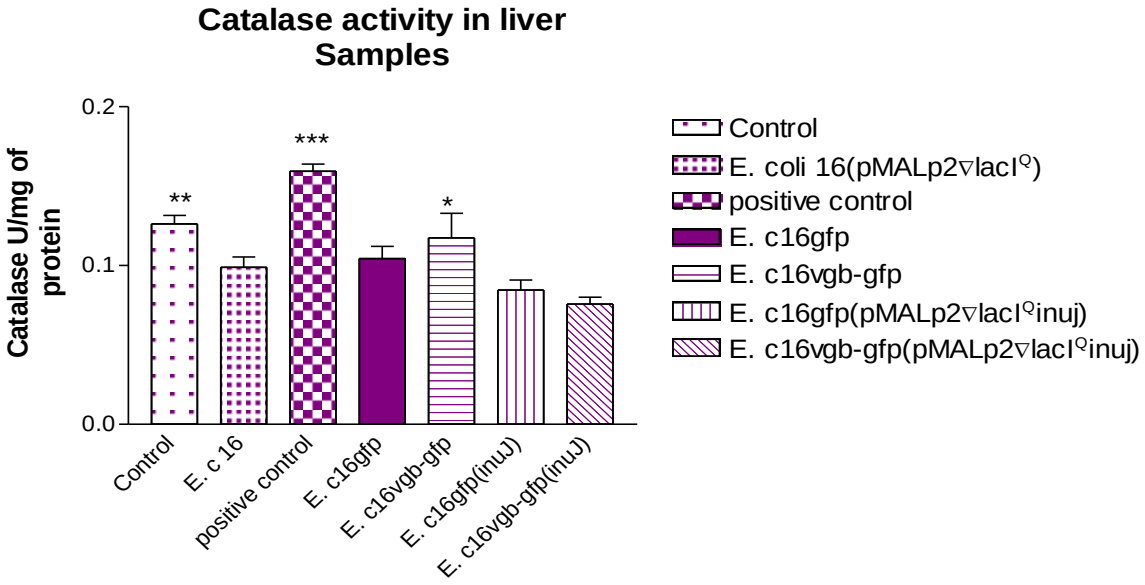
***p<0.001, ** p<0.01 and *p<0.05
Compare to *E. coli* 16
vgb-gfp(pMALp2ΔlacI^{Q}*inuJ*) group



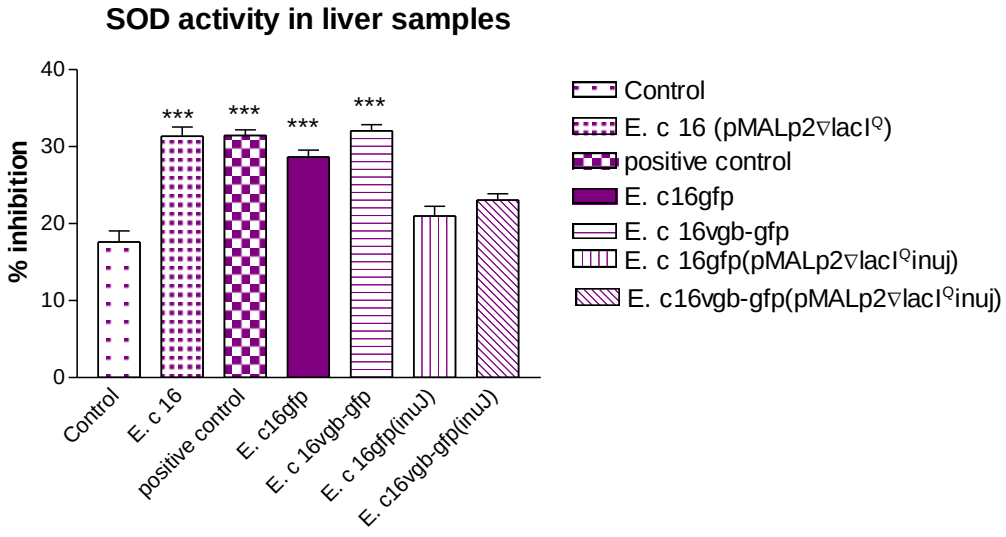
5.3.6.2 Antioxidant status of liver

Antioxidant parameters in liver tissue samples were monitored by the levels of catalase, superoxide dismutase and lipid peroxidation. Catalase activity was 2.1 fold higher in positive control group, 1.6 fold higher in control group compared to inulosucrase groups (E. c 16 *vgbgfp* pMALp2 Δ*lacI*^{Q}-*inuJ*). **Fig 5.17**. SOD activity was 1.36 fold higher in positive control group, 0.76 fold in control group compared to inulosucrase group (E. c 16 *vgbgfp* pMALp2Δ *lacI*^{Q}-*inuJ*). No significant changed where observed in lipid peroxidation.

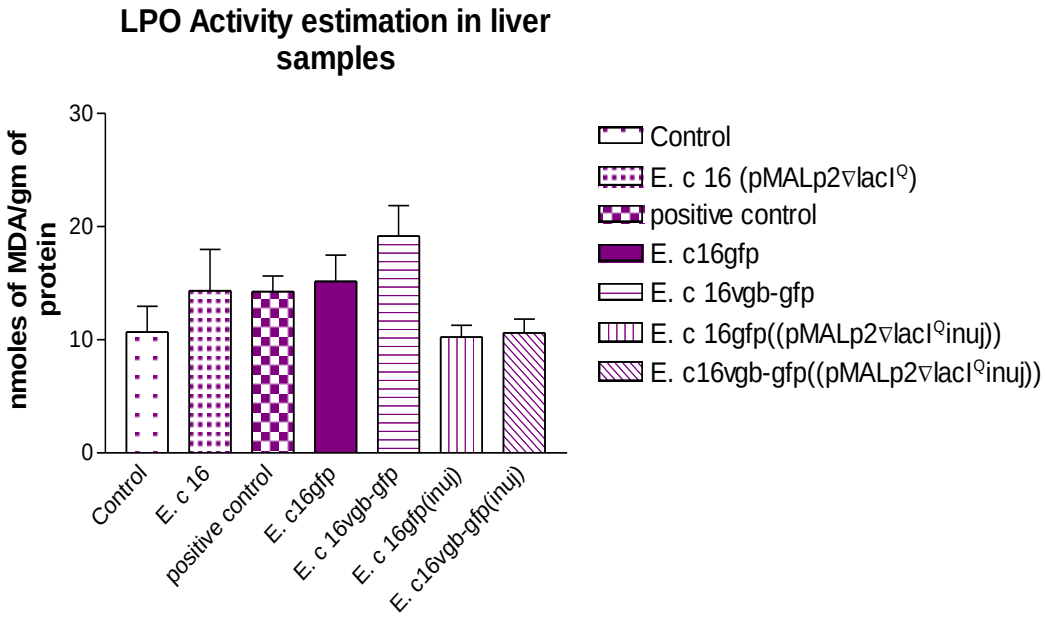
Fig. 5.17. Antioxidant Status of liver tissue in rats fed sucrose diets for 28 days



***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16
vgbgfp(pMALp2▽lacI^{Q}inuJ) groups



***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16
vgbgfp(pMALp2▽lacI^{Q}inuJ) groups

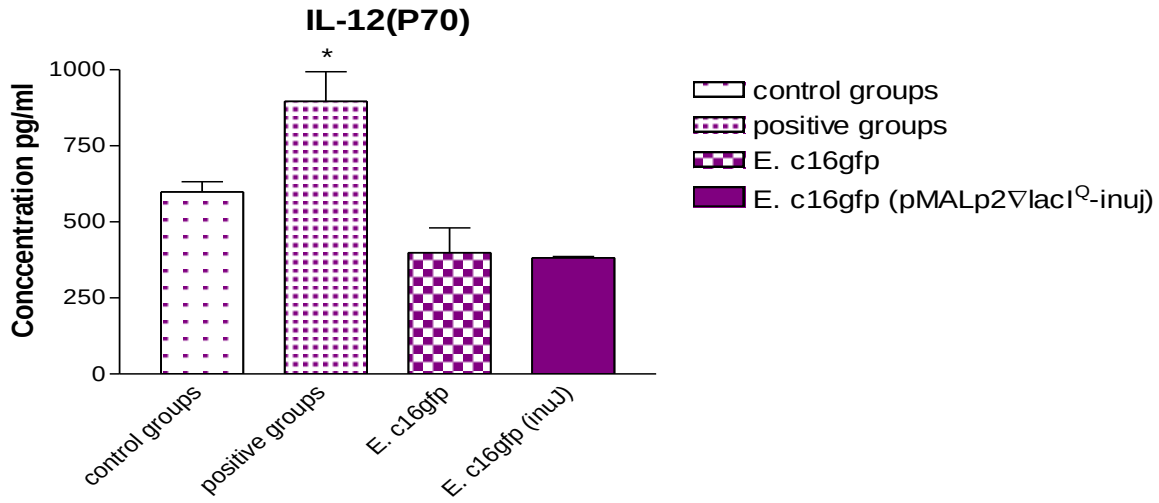


***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16
vgbgfp(pMALp2▽lacI^QinuJ) groups

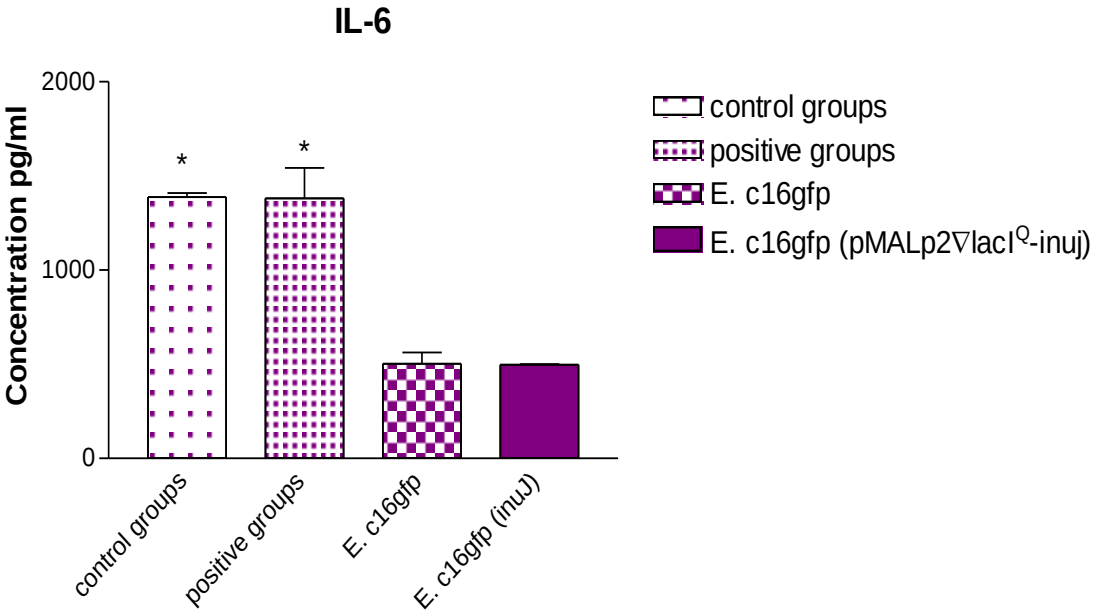
.3.6.3. Cytokine estimation in intestinal tissue samples of selected groups.

Pro-inflammatory cytokines like IL-12, IL-6, IFNγ, IP-10 and Rantes were estimated in selected groups. The levels of IL-12 and IFNγ were significantly increased in positive control groups compared to other groups such as control, *E. c* 16gfp and *E. c* 16gfp (*inuJ*) groups ($p < 0.05$) whereas control and positive control groups had increase in IL-6 level compared to *E. c* 16gfp and *E. c* 16gfp (*inuJ*) groups (**Fig. 5.18)**. Expression of chemokines such as Rantes and IP-10 were similar in all groups.

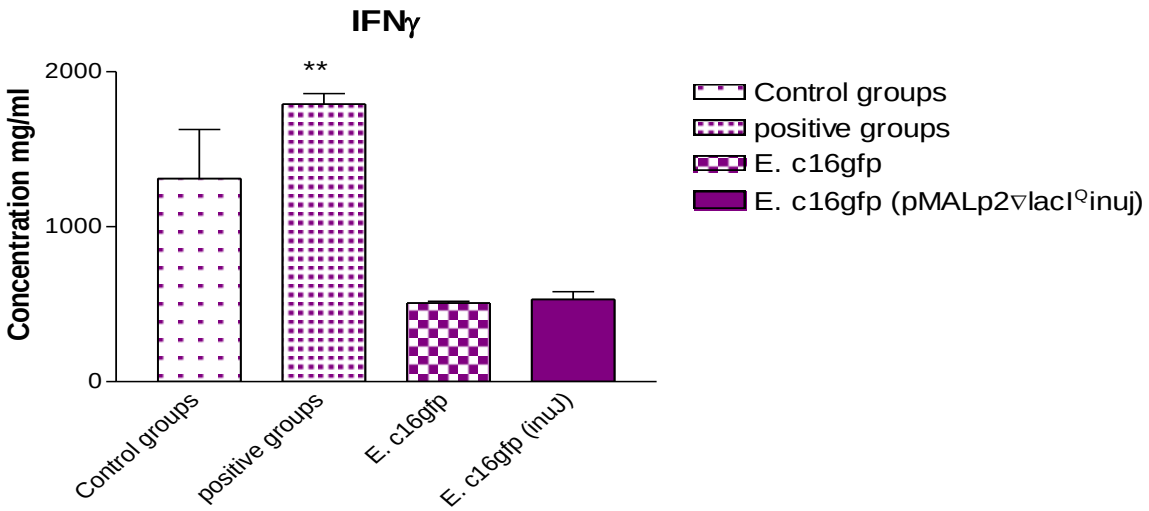
Fig. 5.18. Cytokines level in intestinal tissue of rats fed sucrose diet for 28 days



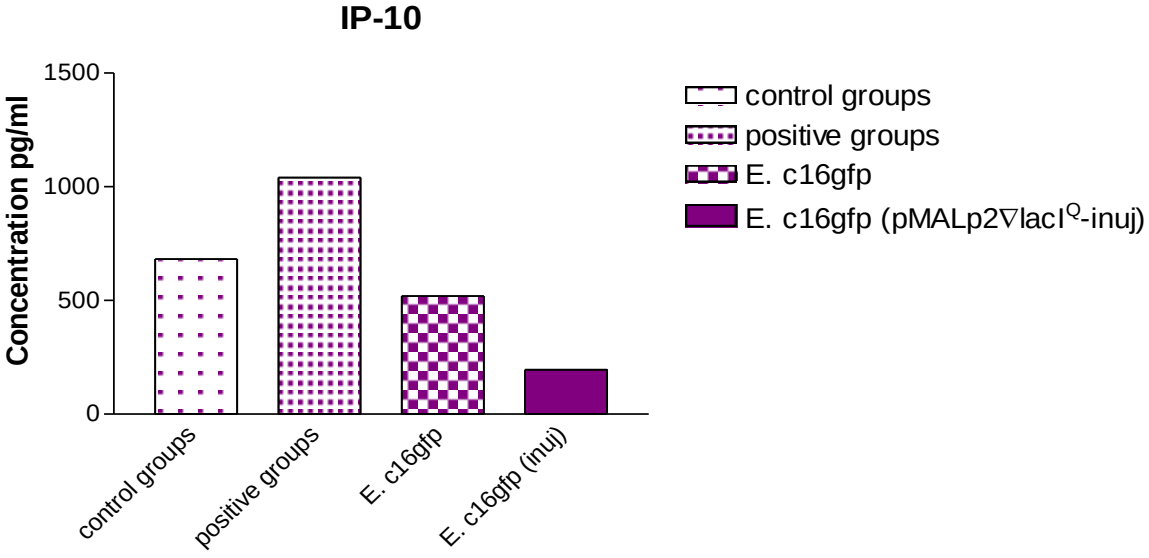
***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16
gfp(pMALp2∇lacI^{Q}inuJ) groups



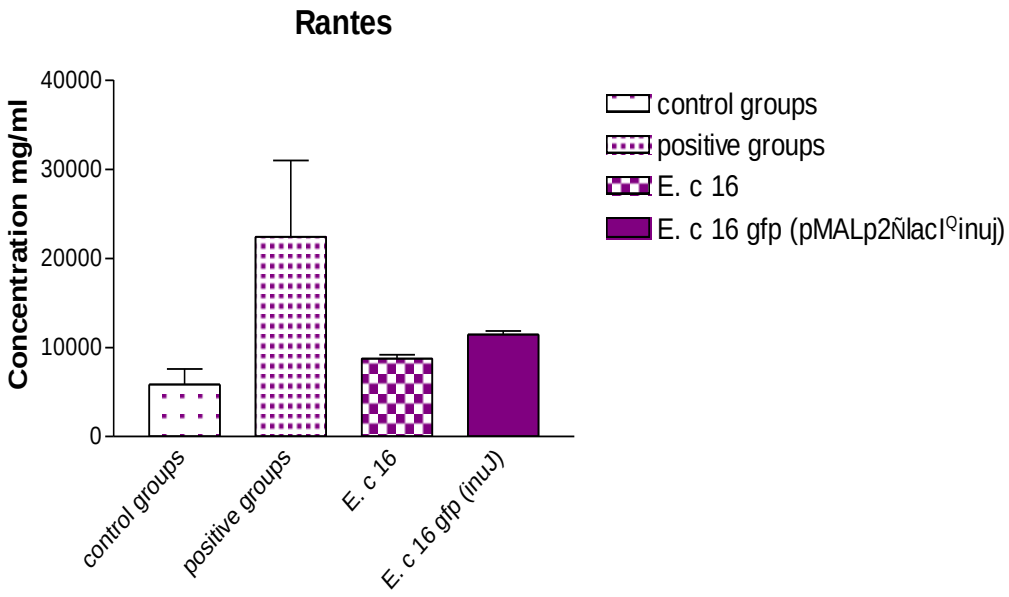
***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16
gfp(pMALp2∇lacI^{Q}inuJ) groups



***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16
gfp(pMALp2▽lacI^{Q}inuJ) groups



20% sucrose as bottle fed to all groups except control groups



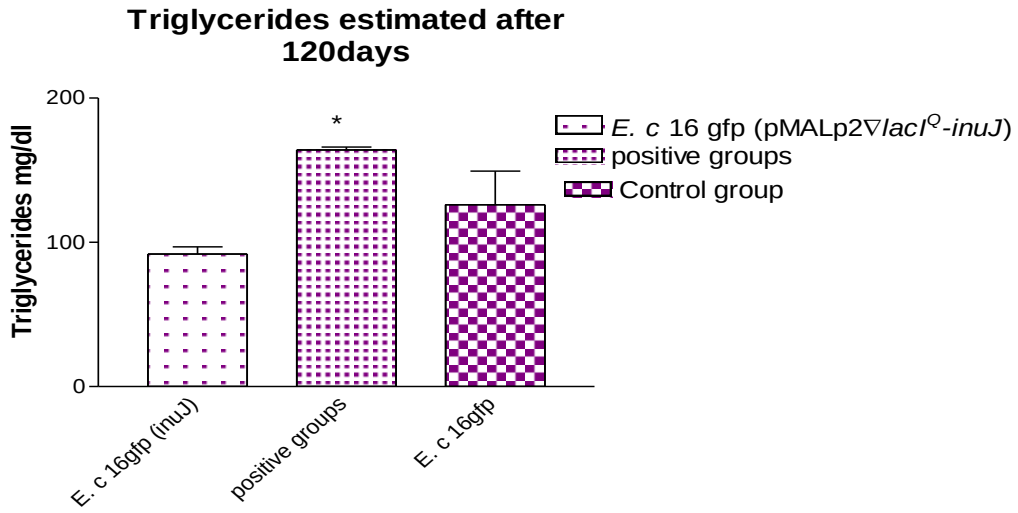
***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16 *gfp*
(pMALp2ΔlacI^{Q}inuJ) groups

5.3.7 Long term effects (120 days) of probiotic harbouring *inuJ* gene. Long term effects of 20% sucrose in drinking water were monitored in three rat groups as mentioned in section 5.2.7.5.

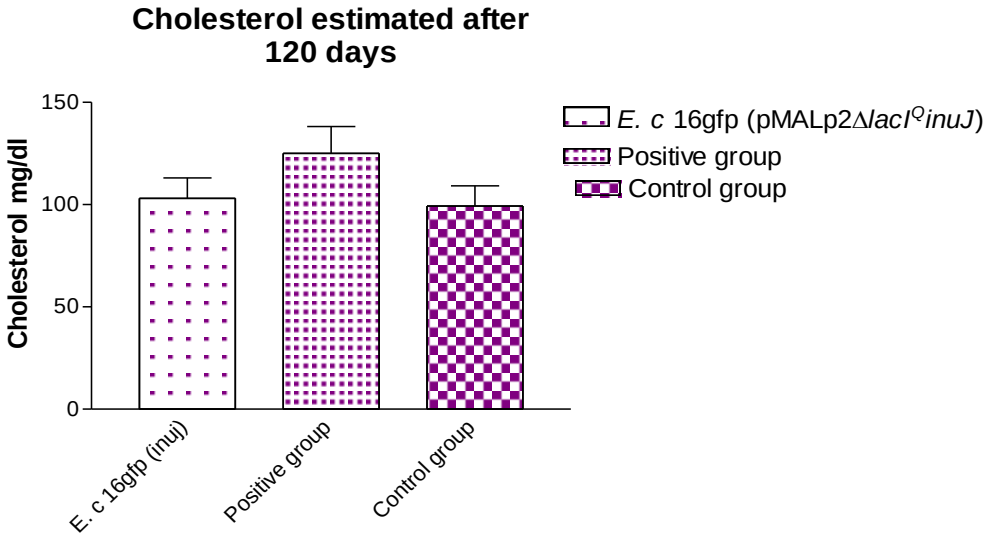
5.3.7.1 Biochemical analysis of serum samples.

A) Lipid profile: Serum TG and LDL cholesterol were significantly lower in InuJ groups (III) compared to positive control groups (II). In Control groups, LDL cholesterol was lower whereas TG was not altered as compared to positive control group (II). HDL was significantly higher in InuJ groups (III) compared to other two group. However, cholesterol showed no significant variation between any groups (**Fig. 5.19**).

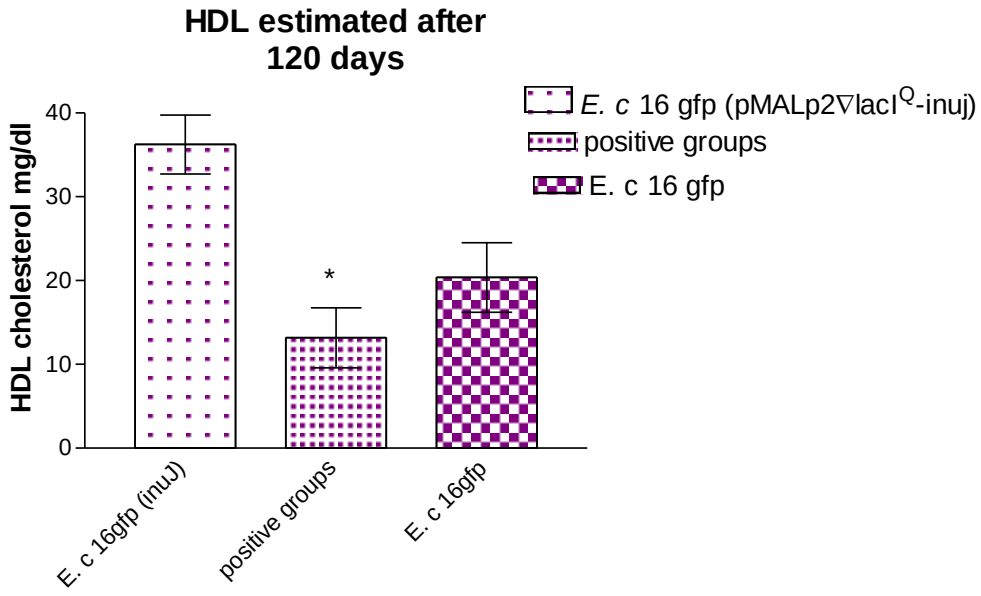
Fig. 5.19. Serum lipid profiles in rats fed sucrose diet for 120 days



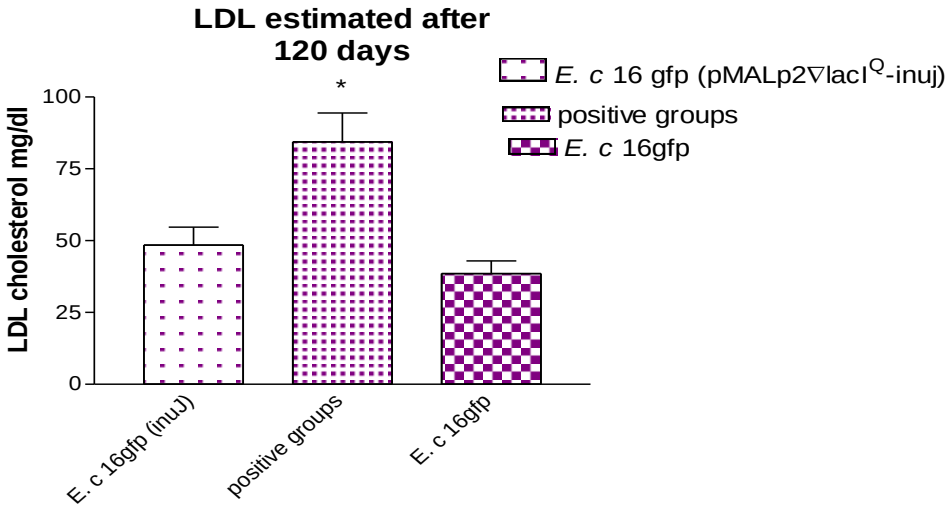
***p<0.001, **p<0.01 and p<0.05
compare to *E. coli gfp*
(pMALp2ΔlacI^{Q}-*inuJ*)



***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16 *gfp*
(pMALp2ΔIacIQ-inuJ)



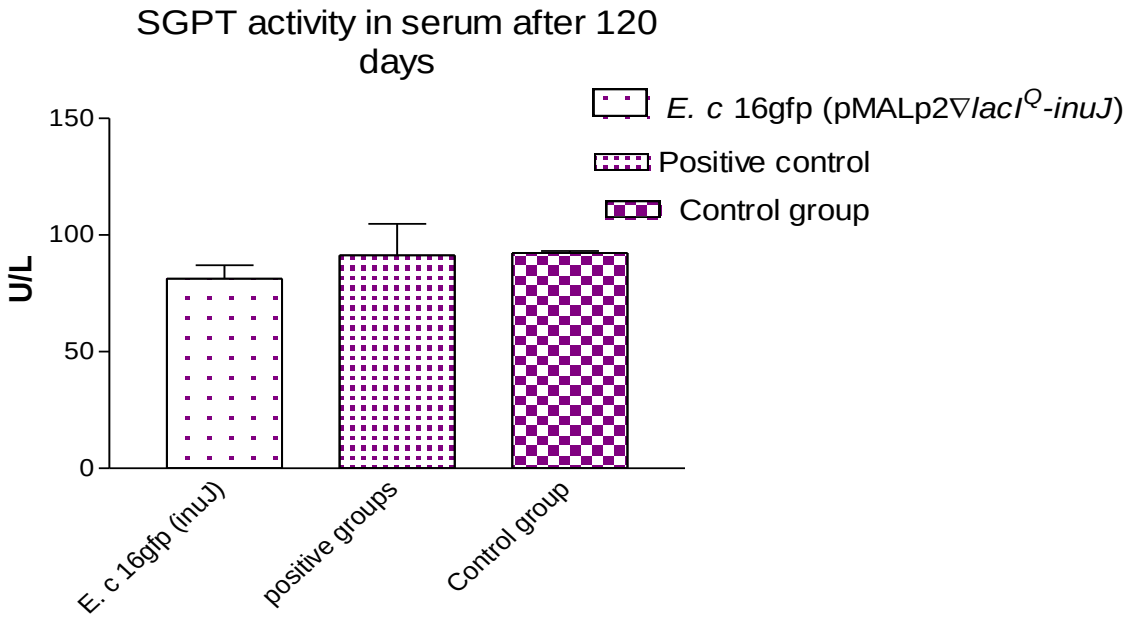
***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16 *gfp*
(pMALp2ΔIacIQ-inuJ)



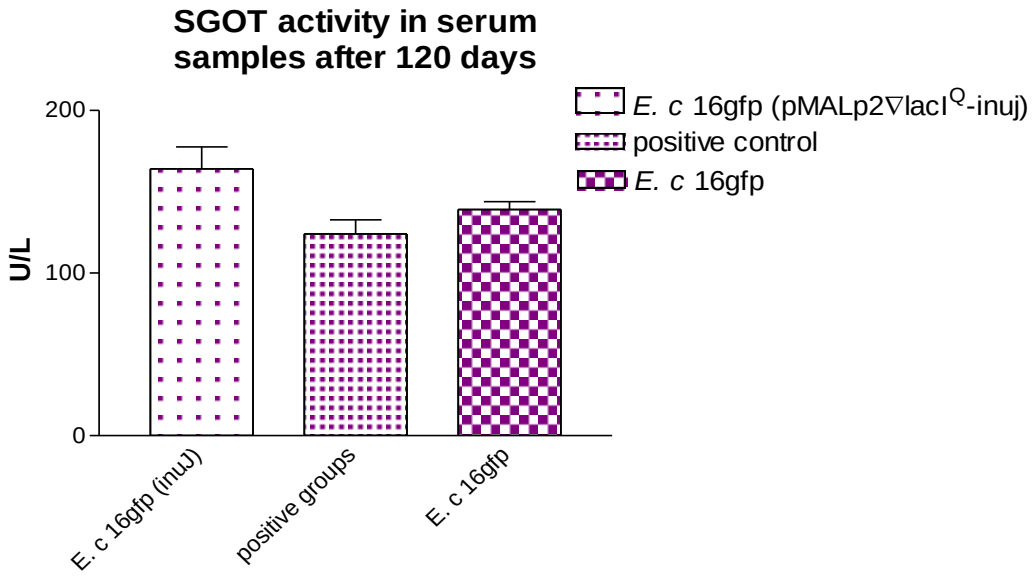
***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16 *gfp*
(pMALp2ΔIacIQ-inuJ)

B) Liver function test. No significant changes were observed in SGPT, SGOT, Alkaline phosphatase activity, total protein, albumin and globulin in all the three groups (**Fig. 5.20**). Total, direct and indirect bilurubin was same in both *E. c* 16 gfp and *E. c* 16 gfp (pMALp2ΔlacI^{Q}-inuj) compare to positive control groups there was significant change was observed ($p < 0.01$).

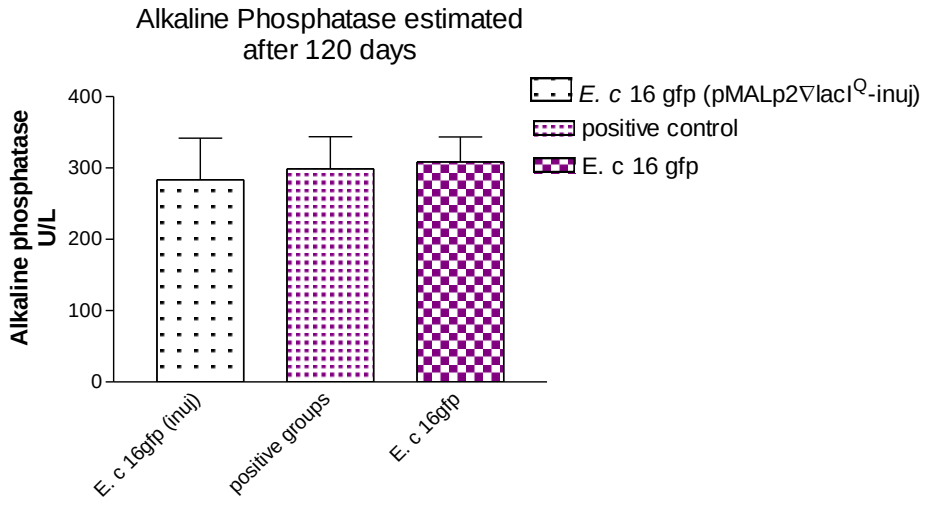
Fig. 5.20. Liver function tests in rats fed sucrose diet for 120 days

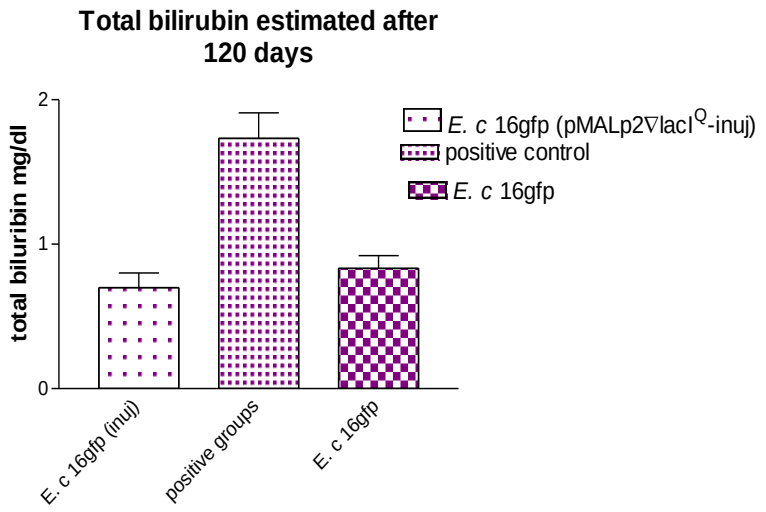


***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16 *gfp*
(pMALp2ΔIacIQ-inuJ)

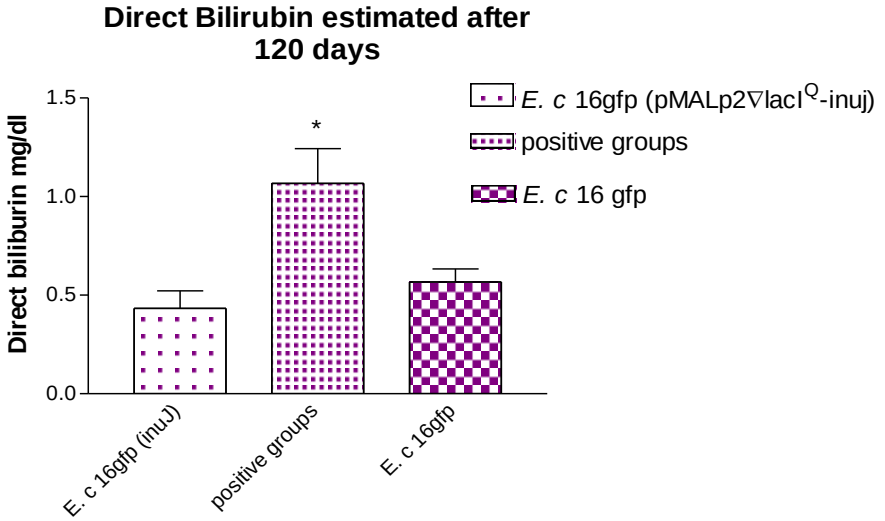


***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16 *gfp*
(pMALp2ΔIacIQ-inuJ)

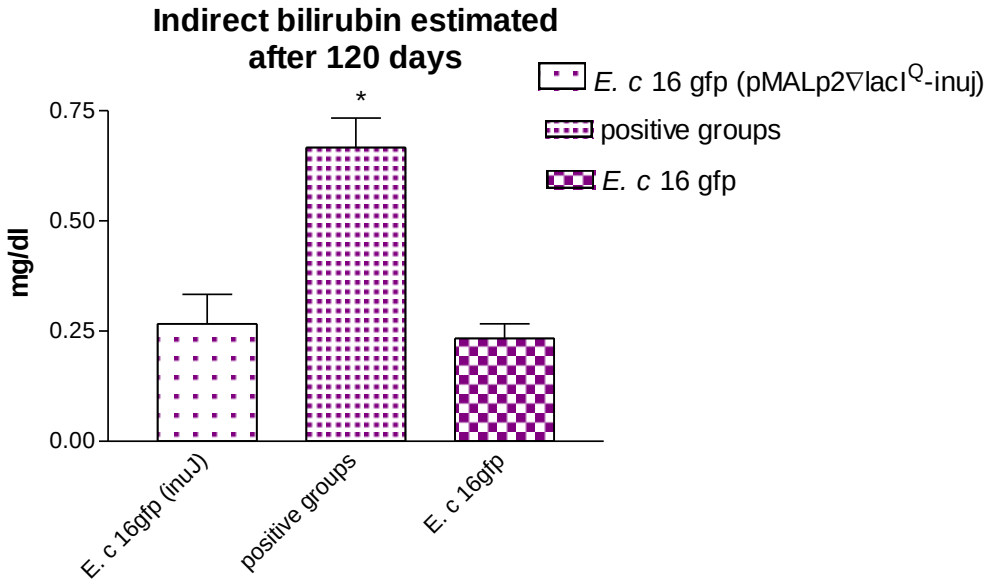




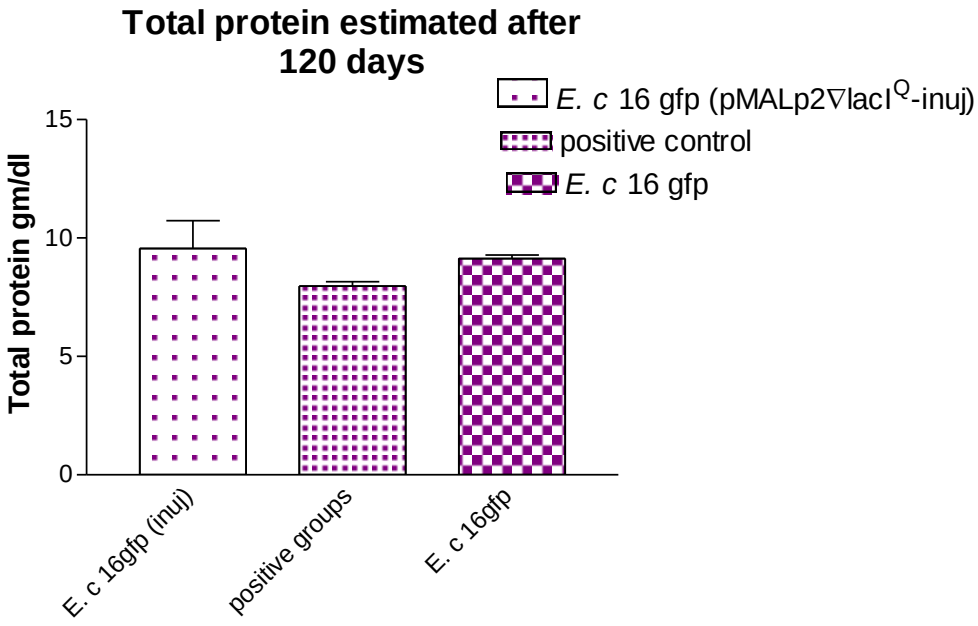
*****p<0.001, ***p<0.01 and p<0.05**
Compare to *E. coli* 16*gfp*
(pMALp2ΔlacI^{Q}-*inuJ*)



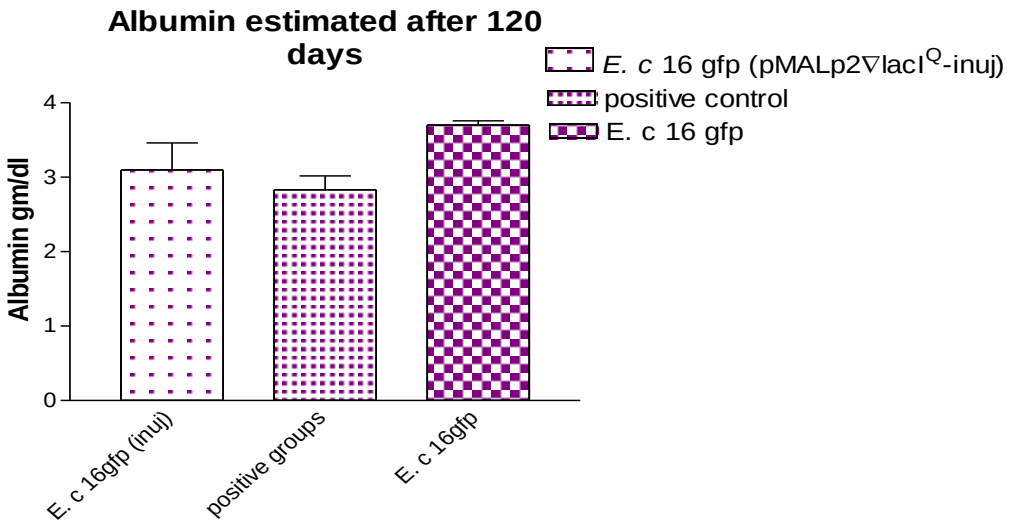
***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16 *gfp*
(pMALp2ΔlacIQ-inuJ)



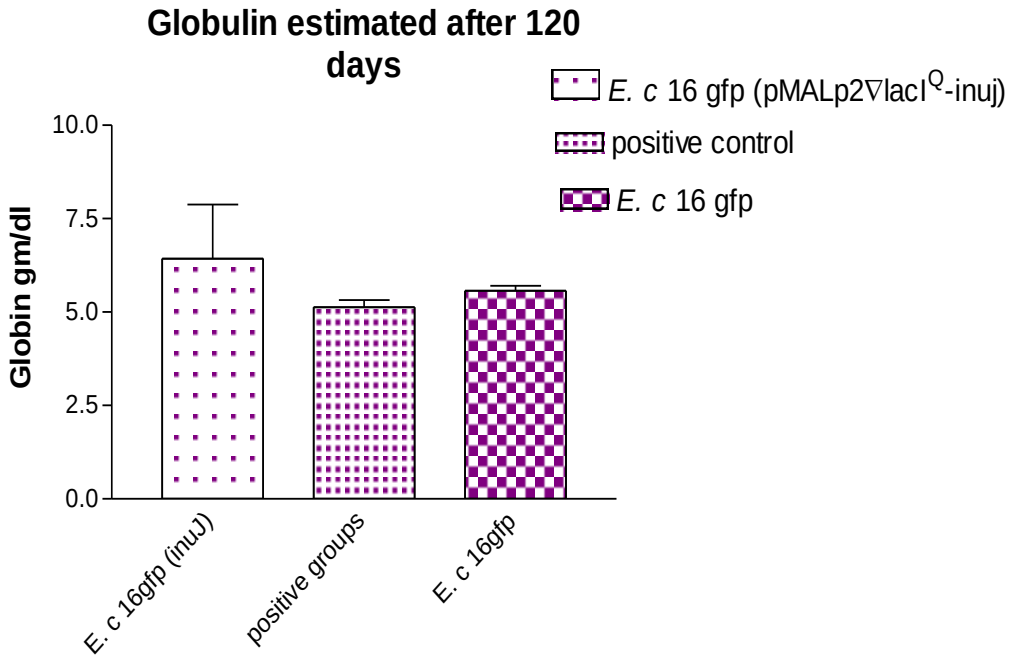
***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16 *gfp*
(pMALp2ΔIacIQ-inuJ)



***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16 *gfp*
(pMALp2ΔIacIQ-inuJ)



***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16 *gfp*
(pMALp2ΔIacIQ-inuJ)



***p<0.001, **p<0.01 and *p<0.05
Compare to *E. coli* 16 *gfp*
(pMALp2ΔIacIQ-inuJ)

5.4. Discussion

Hypertriglyceridemia is correlated with increase in small, dense particles of LDL cholesterol and reduction in HDL cholesterol (Benzer *et al*., 2001). This condition often associated with premature coronary heart disease. High sucrose diet or fructose diet had been used as a model system for four decades to study the effects of hypertriglyceridemia (Cani *et al*., 2007; Sievenpiper *et al*., 2009). Different strategies have been employed to overcome the problem associated with hypertriglyceridemia; one of the strategies presently in use is prebiotic products (Kelly, 2008). The hypolipidemic effect of FOS has been well established (Agheli *et al*., 1998; Fukasawa *et al*., 2009). Prebiotic products like FOS have been shown to decrease metabolic disorders by tweaking the complex interactions that occur in the microbial ecosystem residing in our gut (Delzenne *et al*., 2008). Our short term or long term studies with sucrose diet in rats indicate that *in vivo* production of FOS from sucrose using the InuJ can significantly reduce TG level and increase in HDL level.

The short term effects of 20% sucrose fed to rats significantly increased the catalase and SOD activity indicative of elevated ROS and oxidative stress. Potent antioxidant effects showed by inulosucrase groups might be due to the synergistic effects of both *vgb* and *inuJ* gene. *vgb* gene has shown to possess peroxidase activity (Kvist *et al*., 2007; Ayudhya *et al*., 2010).

Inflammation in mucosal surface of intestinal tissue is initiated and perpetuated by the secretion of proinflammatory cytokines and chemokines. Probiotic *E. c* 16 with or without inulosucrase acts as potent anti-inflammatory as compared to positive control groups. The long term 20% sucrose fed rats showed similar results to the short term studies. Long term studies showed high level increase in LDL (bad) cholesterol with concomitant decrease in HDL (good) cholesterol. The condition clearly manifests premature coronary heart disease which were absent in InuJ group.

Present study demonstrated that probiotic *E. coli* 16 secretes InuJ into the medium but not in *E. coli* BL 21 strain which had been attributed to the antimicrobial colicin secretion. *E. coli* 16 secretes colicin E1 & Ib (Prasant *et al*., 2009) whereas *E. coli* BL21 does not secret any colicins. This observation was supported by the fact that the extracellular target protein coexpressed with bacteriocin release protein was used to release periplasmic proteins into the culture medium (Sommer *et al*., 2010). Probiotic *E. coli* 16 harboring *inuJ* gene secretes InuJ enzyme which in turn efficiently converts sucrose to FOS. Thus, genetically engineered probiotic *E. coli* 16 acts as a synbiotic.

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