

## CHAPTER 5

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

## 5.1 Introduction

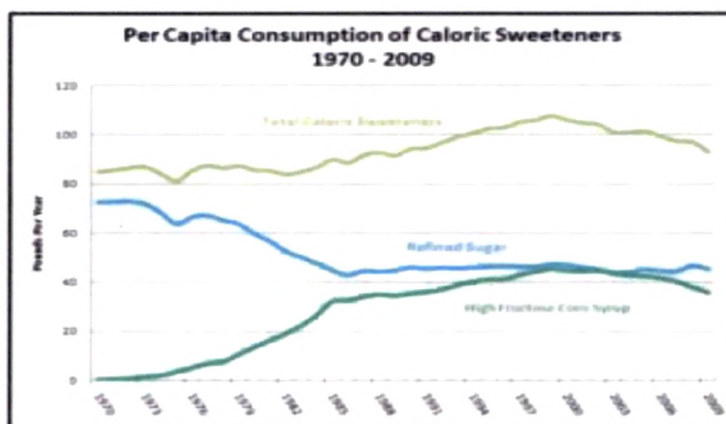
Obesity has reached epidemic proportions globally. In 2007, it was estimated that more than 1.1 billion adults were overweight and at least 312 million of them clinically obese - and are major contributor to the global burden of chronic disease and disability (Hossain *et al.*, 2007). Over the past 20 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).

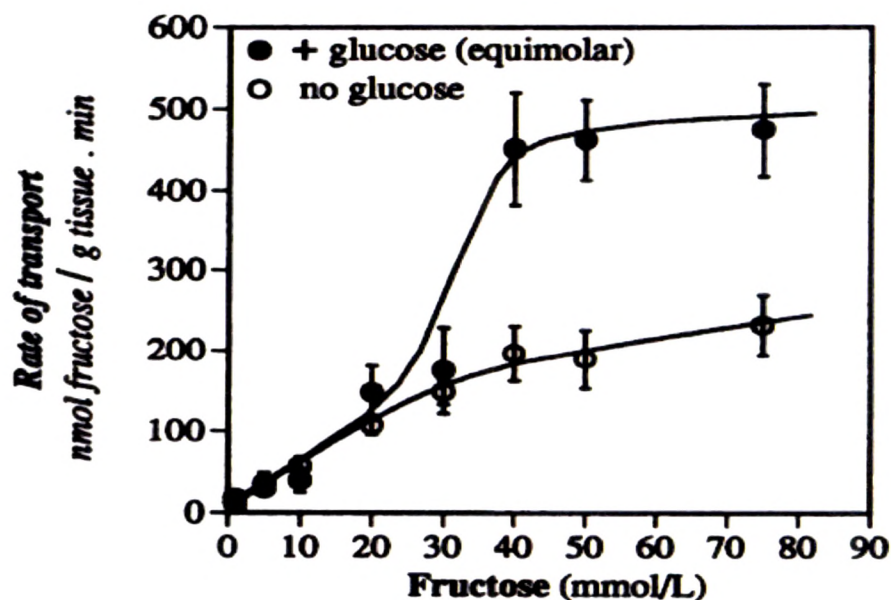
**Fig. 5.1: Per capita consumption of calorific sweeteners (U.S. department of agriculture, Economic research service, 2010)**



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

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

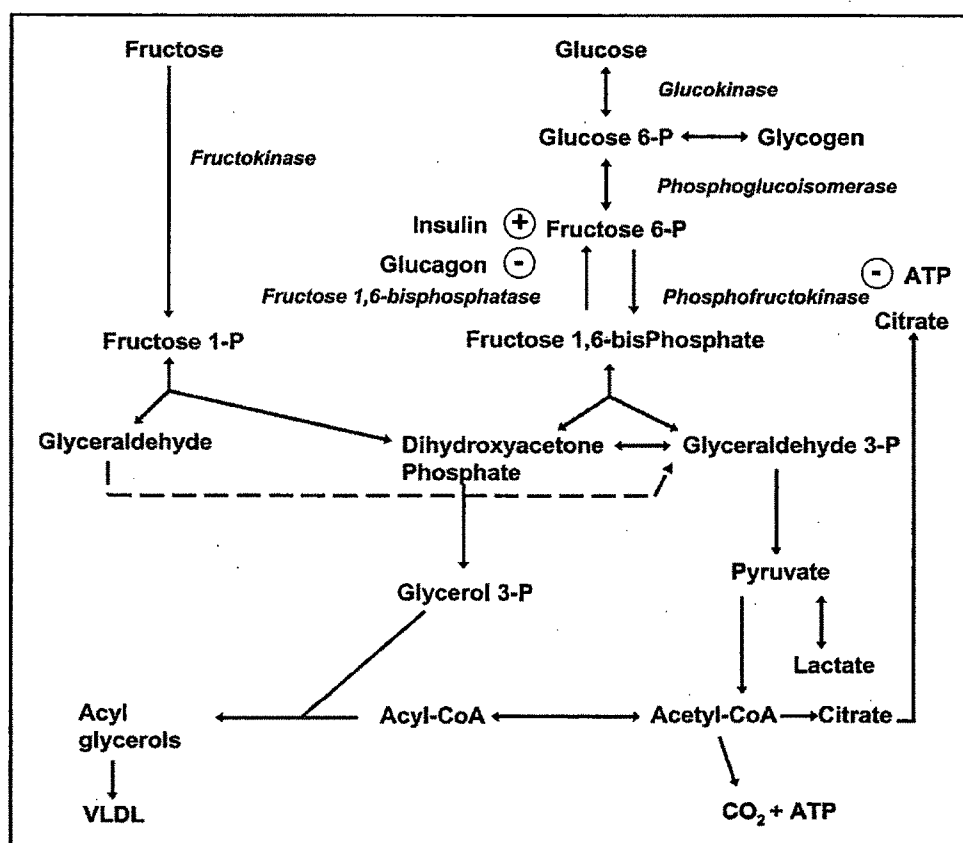
Fig. 5. 2: Fructose absorption in presence and absence of glucose (Berghe *et al.*, 1986)



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 aldolase. 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)



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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 polysaccharides), 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 digestible 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  $\beta$ -2,1 position of sucrose by the transfructosylating enzymes like glucosyltransferase, fructosyltransferase and inulosucrase (Yun, 1996). The  $\beta$ -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.

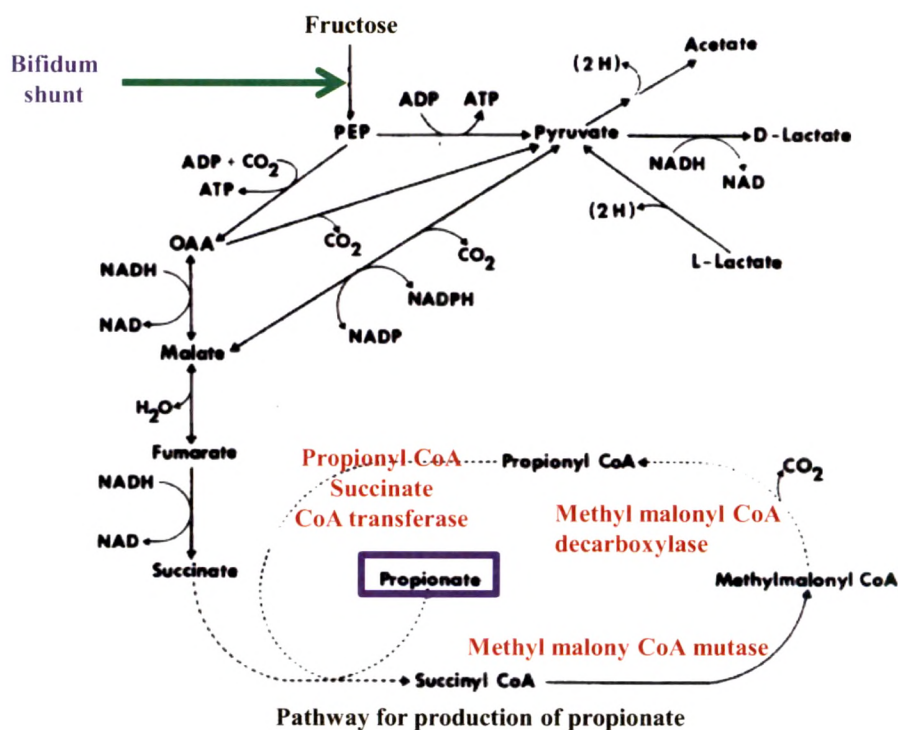
FOS acts as a soluble fiber and can be used in increasing intestinal motility, transport and reduction of high levels of plasma cholesterol (Posada *et al.*, 2002). FOS can decrease triacylglycerol and very low density lipoprotein (VLDL) when given to rats. The triacylglycerol lowering action is due 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 (Crittenden, 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 cholesterologenesis 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 cholesterologenesis 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



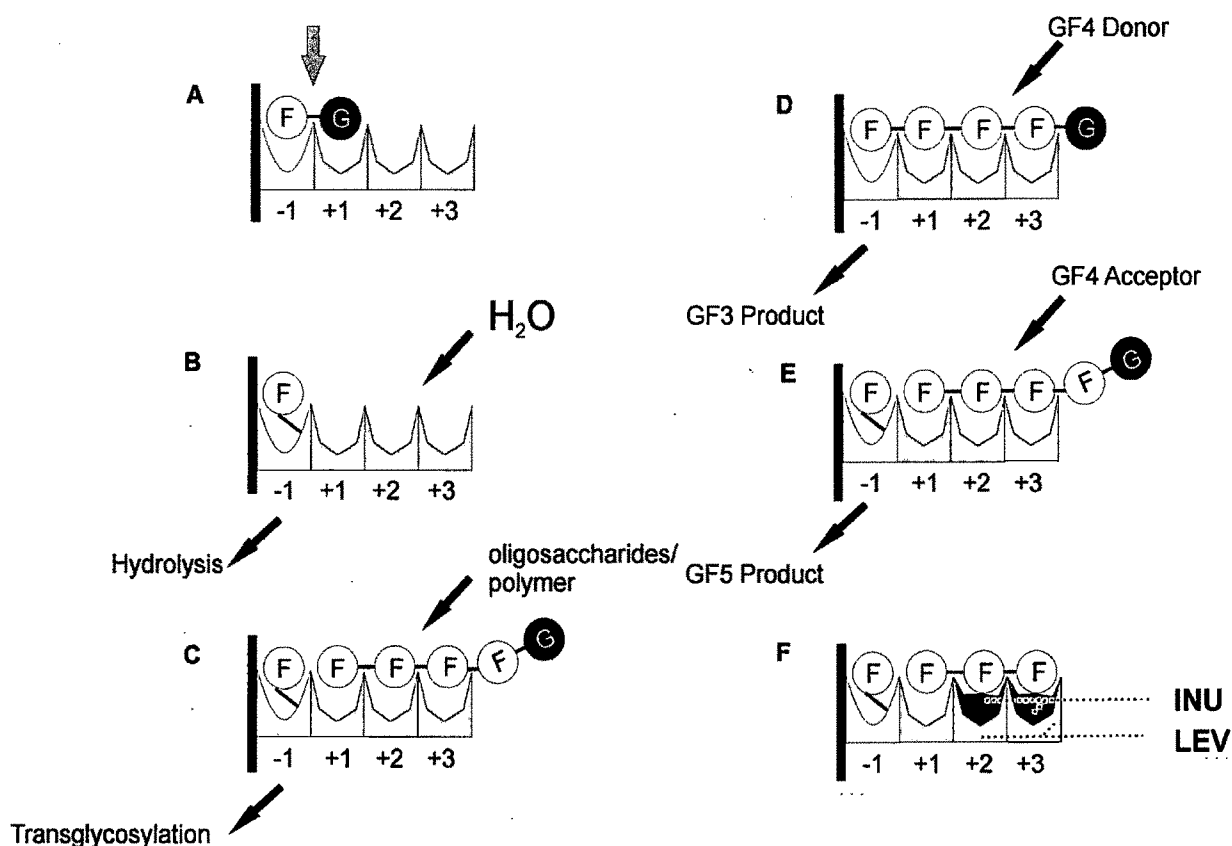
and VLDL in rats fed with FOS (Delzenne *et al.*, 2008). Feeding of FOS increased *Bifidobacterium* spp., SCFA concentrations, decrease *Fusobacterium* spp. and pH (O'Sullivan *et al.*, 1996; Fuller *et al.*, 1998). *Bifidobacterium* spp. selectively ferment FOS in preference to other carbohydrate sources like starch, fructose, pectin and polydextrose (Gibson *et al.*, 1995). FOS also reduces the expression of genes coding Fatty Acyl 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 (PPAR $\alpha$ ). However, PPAR $\alpha$  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  $\beta$ -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* spp.



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  $\beta$ -linked glycosidic bonds with net retention of the anomeric configuration and / or hydrolysis. These FTF activities result in: (i) Transglycosylation 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  $\beta$ -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  $\beta$  (2-6) linked fructose units (levan) and only limited information is available about bacterial inulosucrases (InuJ; EC 2.4.1.9) producing  $\beta$  (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 *ImuJ* and *Lev* is reflected by a shallow (dark grey) (low affinity) and deep cleft (light grey) (high affinity), respectively (F). Sugar binding sub-

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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 where 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* DH5 $\alpha$  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<sub>2</sub>HPO<sub>4</sub>·7H<sub>2</sub>O, 3 g/l KH<sub>2</sub>PO<sub>4</sub>, 0.5 g/l NaCl, 1 g/l NH<sub>4</sub>Cl, 3 mg/l CaCl<sub>2</sub>, 1 mM MgSO<sub>4</sub>, thiamine and trace elements. Antibiotics were used at the following final concentrations: ampicillin 100  $\mu$ g ml<sup>-1</sup>. Plasmid pET15b-*inuJ* was a generous gift from Dr. Dijkhuizen. *E. coli* DH5 $\alpha$  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  $\mu$ g/ml ampicillin.

### 5.2.2. Construction of plasmids: pGRG-*gfp*, pGRG 8-16*gfp*, pMAL-p2 $\Delta$ lacI<sup>Q</sup> and pMAL-p2 $\Delta$ lacI<sup>Q</sup>-*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 MluI/EcoRV, end filled and further self ligated, disrupting the *lacI*<sup>Q</sup> gene to obtain constitutive pMAL-p2 $\Delta$ lacI<sup>Q</sup> vector. Confirmation of disrupted clones of pMAL-p2 $\Delta$ lacI<sup>Q</sup> 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-p2 $\Delta$ lacI<sup>Q</sup> 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°C. Grown culture was streaked on LA plate containing L-arabinose (0.1%) without ampicillin to induce *tns* genes, followed by incubation at 42°C 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- <i>gfp</i>	derived from the high-copy number vector pUC18 by insertion of a modified <i>gfp</i> gene; Ap <sup>r</sup>	Schultz et al. 2005
pUC8:16	derived from the high-copy number vector pUC8 by insertion of a <i>vgb</i> gene; Ap <sup>r</sup>	Stark et al. 1994
pET15b- <i>inuJ</i>	Expression vector, derived from pET15b by insertion of a inulosucrase <i>inuJ</i> gene; Ap <sup>r</sup>	Dijkhuizen et al. 2008
pUC8:16- <i>gfp</i>	derived from the high-copy number vector pUC8:16 by insertion of a modified <i>gfp</i> gene; Ap <sup>r</sup>	This study
pGRG- <i>gfp</i>	derived from low copy no. integration vector pGRG-36 by insertion of a modified <i>gfp</i> gene; Ap <sup>r</sup>	This study
pGRG 8-16 <i>gfp</i>	derived from low copy no. integration vector pGRG-36 by insertion of a <i>vgb</i> and modified <i>gfp</i> gene; Ap <sup>r</sup>	This study
pMAL-p2Δ <i>lacI</i> <sup>Q</sup>	deletion of <i>lacI</i> <sup>Q</sup> from periplasmic expression vector pMal-p2; Ap <sup>r</sup>	This study
pMAL-p2Δ <i>lacI</i> <sup>Q</sup> - <i>inuJ</i>	derived from periplasmic expression vector pMAL-p2Δ <i>lacI</i> <sup>Q</sup> by insertion of <i>inuJ</i> ; Ap <sup>r</sup>	This study
Bacterial strains		
<i>E. coli</i> BL21	F <sup>+</sup> <i>ompT hsdSB (rB- mB-) gal dcm</i>	Sambrook and Russell. 2001
<i>E. coli</i> DH5α	F- <i>endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ80dlacZΔM15Δ(lacZYA-argF)U169, hsdR17(rK- mK+), λ-</i>	Sambrook and Russell. 2001
<i>E. coli</i> isolate16	Wild type	Prasant et al. 2009

#### 5.2.4. Transformation of pMAL-p2ΔlacI<sup>Q</sup> and pMAL-p2ΔlacI<sup>Q</sup> -*inuJ* plasmid in *E. coli* 16 integrants.

The pMAL-p2ΔlacI<sup>Q</sup> and pMAL-p2ΔlacI<sup>Q</sup>-*inuJ* plasmids were independently transformed into *E. coli* 16-*gfp* chromosomal integrants using the CaCl<sub>2</sub> method. Similarly, plasmids pMAL-p2ΔlacI<sup>Q</sup> and pMAL-p2ΔlacI<sup>Q</sup> -*inuJ* were also independently transformed into *E. coli* 16-*vgb-gfp* chromosomal integrants using the CaCl<sub>2</sub> method. Transformants were selected on LA plates containing ampicillin 100μg ml<sup>-1</sup>.

#### 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-p2ΔlacI<sup>Q</sup> and pMAL-p2ΔlacI<sup>Q</sup> -*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)

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gels were incubated at ambient temperature in McIlvaine'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 (Rudolph *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 ( $21 \pm 2$  °C). 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. *In vivo* 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 ( $5 \text{ gml}^{-1}$ ) 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 (%).**

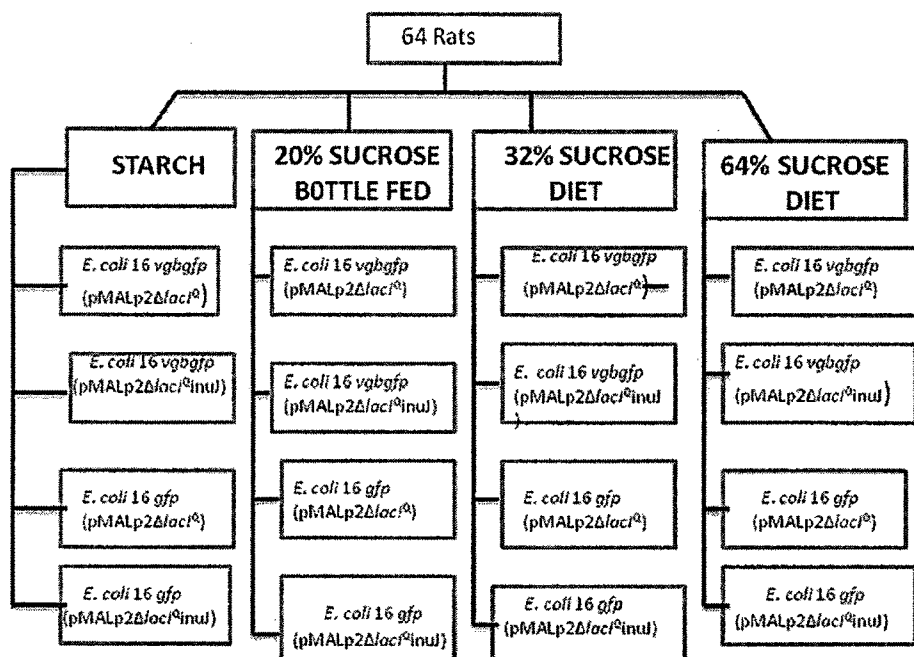
The animals were divided into four groups of sixteen rats each. Group 1, control animals (Starch), received the control diet containing 64 percent starch and tap water *ad libitum* [64% starch, 20% casein, 0.7% methionine, 5% groundnut oil, 9.7% wheat bran and 3.5% salt mixture (The mineral mix in a kg contained  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 30.5 g.; NaCl, 65.2 g.; KCl, 105.7 g.;  $\text{KH}_2\text{PO}_4$ , 200.2 g.;  $\text{MgCO}_3$ , 3.65g.;  $\text{Mg}(\text{OH})_2 \cdot 3\text{H}_2\text{O}$ , 38.8 g.;  $\text{FeC}_6\text{H}_5\text{O}_7 \cdot 5\text{H}_2\text{O}$ , 40.0 g.;  $\text{CaCO}_3$ , 512.4 g.; KI, 0.8 g.; NaF, 0.9 g.;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 1.4 g.;



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MnSO<sub>4</sub>, 0.4 and CONH<sub>3</sub>, 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 fecal 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	<i>E. coli</i> 16	Chow diet
Group III (Positive control group)	-	Chow diet with 20% sucrose
Group IV	<i>E. coli</i> 16gfp	Chow diet with 20% sucrose along with weekly doses of <i>E. coli</i> 16gfp
Group V	<i>E. coli</i> 16vgb-gfp	Chow diet with 20% sucrose along with weekly doses of <i>E. coli</i> 16vgb-gfp
Group VI	<i>E. c</i> 16gfp (pMALp2ΔlacIQinuj)	Chow diet with 20% sucrose along with weekly doses of <i>E. coli</i> 16vgb-gfp
Group VII	<i>E. c</i> 16vgb-gfp (pMALp2ΔlacIQinuj)	Chow diet with 20% sucrose along with weekly doses of <i>E. coli</i> 16vgb-gfp

### **5.2.8. Biochemical parameters estimation.**

#### **5.2.8.1. Biochemical analyses in serum samples**

SGOT, SGPT, bilirubin, 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°C 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 ( $\mu\text{mol/mg}$  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. Cytokine 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  $\text{KH}_2\text{PO}_4$  and 0.115 g of  $\text{Na}_2\text{HPO}_4$  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**

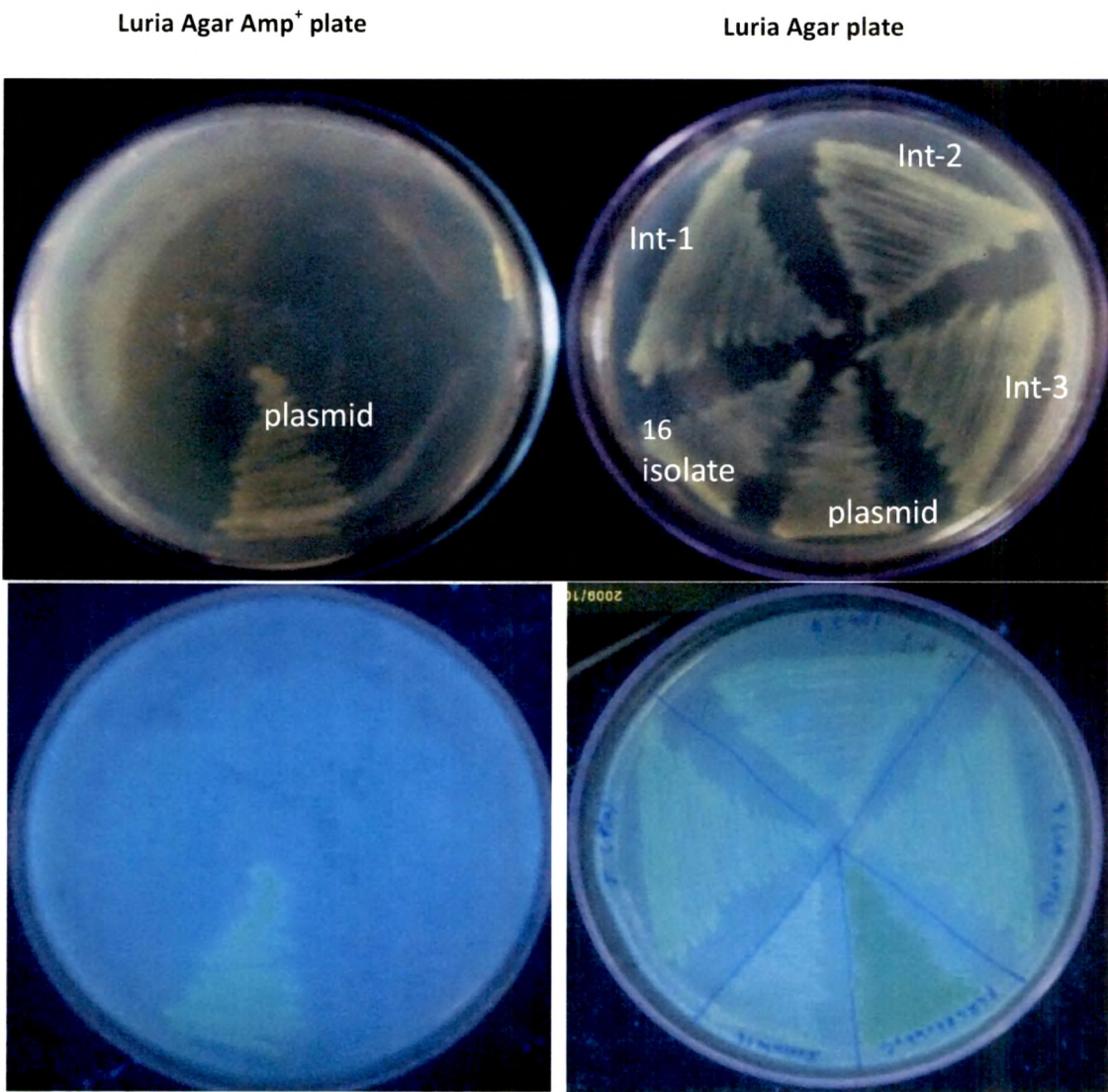
Statistical evaluation of the data was done by one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison test and results were expressed as mean  $\pm$  SEM using Graph Pad Prism version 3.0 for Windows, Graph Pad Software, San Diego, California, USA.

5.3. Results

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

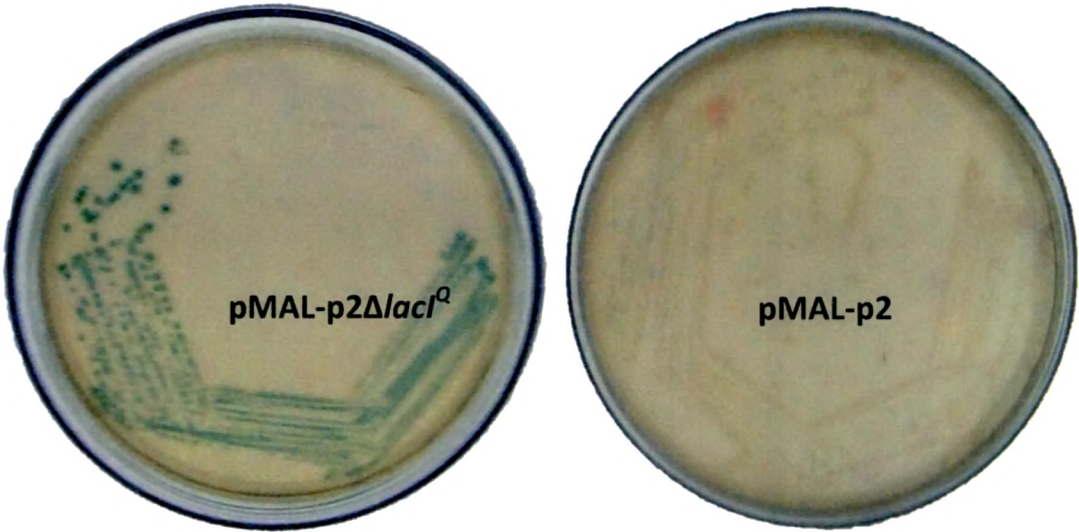
The *E. coli* 16 integrant (pGRG-*gfp* and pGRG *vgb-gfp*) showed loss of the antibiotic resistance and lesser fluorescence compared to the plasmid transformants which was accounted by the fact that the integrant single copy number (**Fig. 5.6**).

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



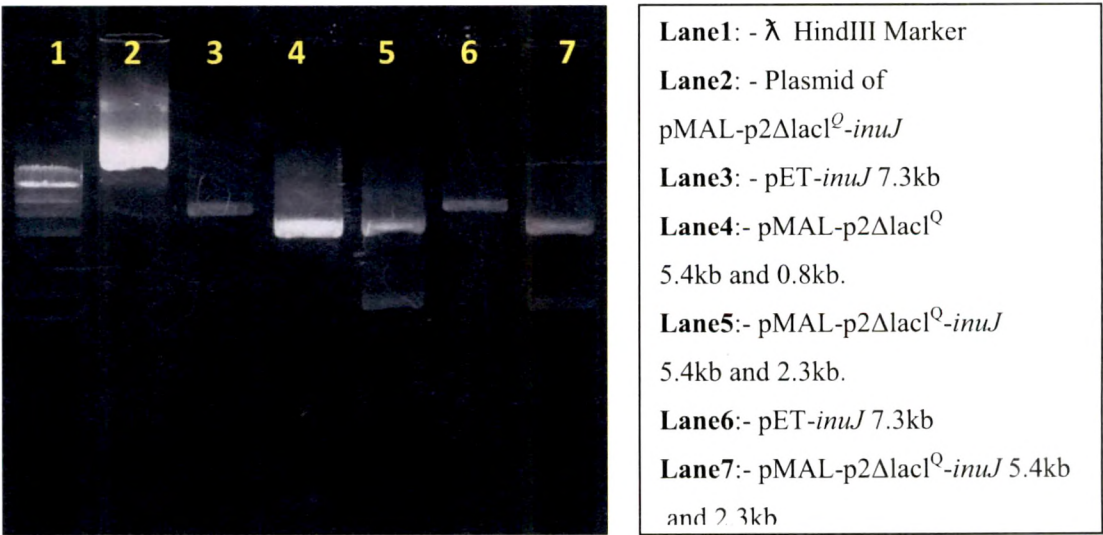
*E. coli* 16 integrants harboring pMAL-p2Δ*lacI*<sup>Q</sup> plasmid showed constitutive phenotype on X-gal plate due to disruption of *lacI*<sup>Q</sup> repressor binding protein site (Fig. 5.7).

Fig. 5.7: Phenotypic confirmation of pMAL-p2Δ*lacI*<sup>Q</sup> clones.



Insertion of inulosucrase gene in pMAL-p2Δ*lacI*<sup>Q</sup> was confirmed by release of 2.3 kb fragment upon digestion with *S*all and *B*glII enzymes (Fig. 5.8).

Fig. 5.8: Restriction digestion pattern for pMAL-p2Δ*lacI*<sup>Q</sup>-*inuJ* plasmids

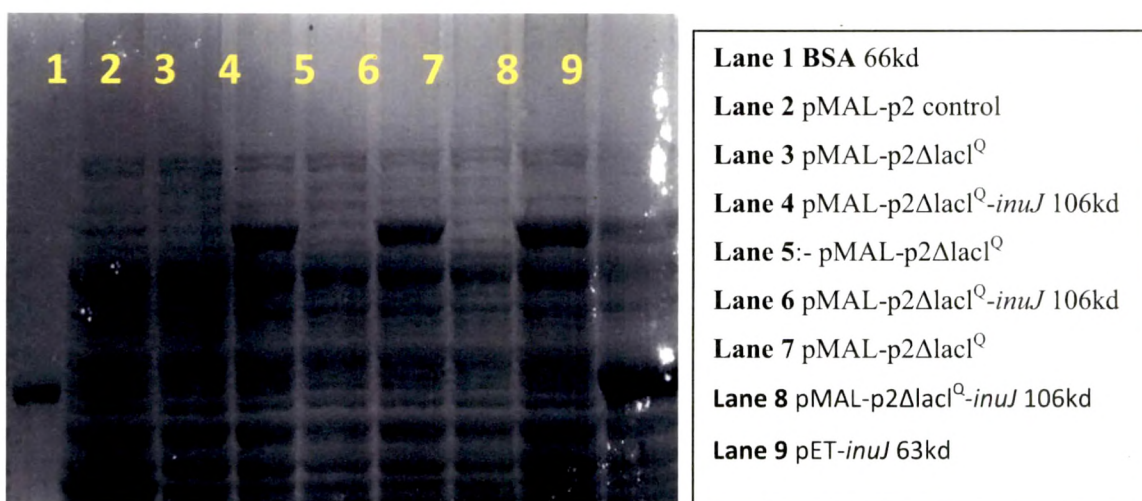






SDS PAGE of cell free extract of *E. coli* BL21 (DE3) containing InuJ showed the presence of 63 kD protein but in the clones a band was seen at 106 kD region. This is because InuJ protein is obtained as MBP-InuJ as a translational fusion protein with a molecular weight of 106 kD (Fig. 5. 9).

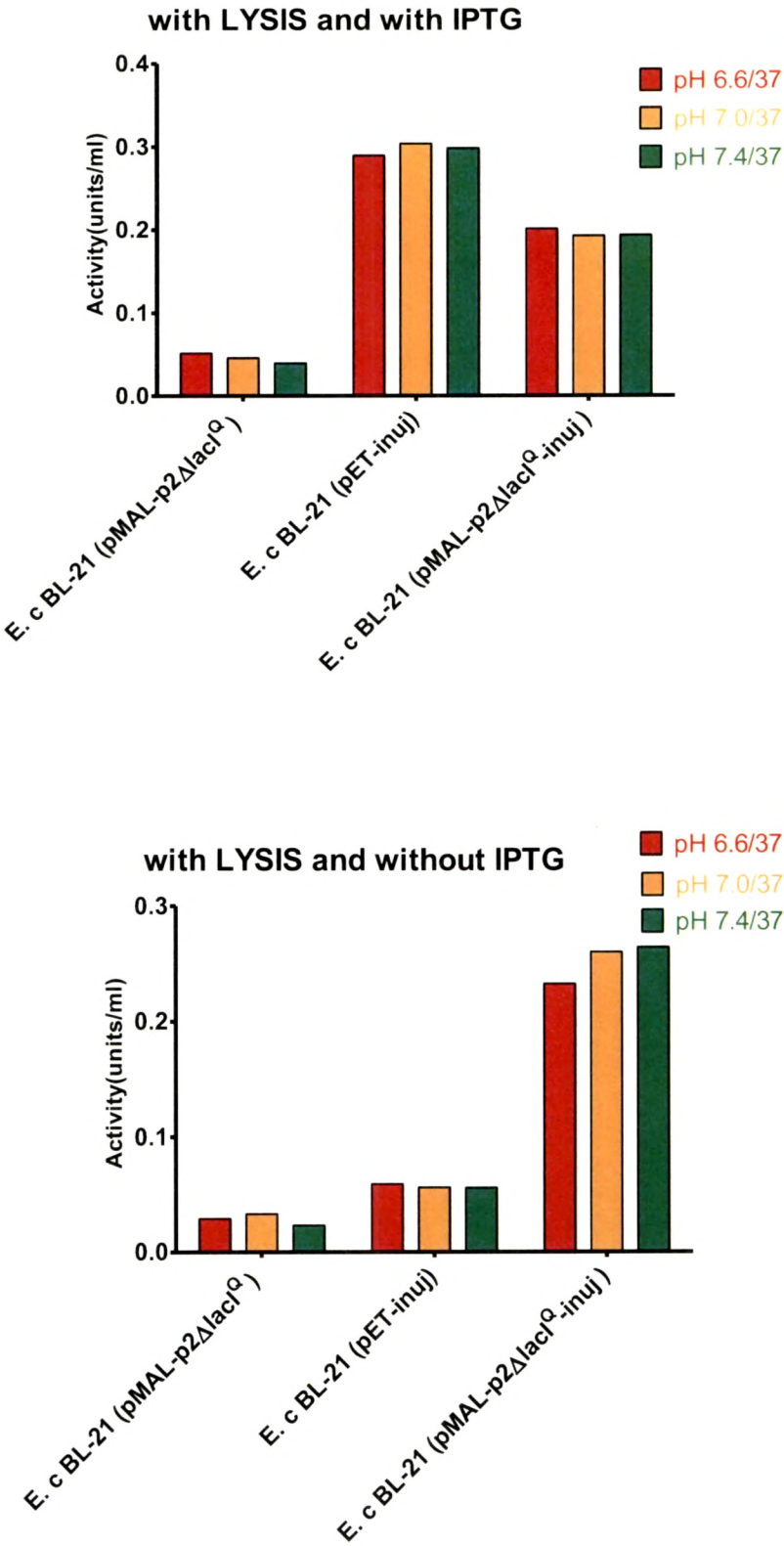
**Fig. 5.9: SDS-PAGE analysis of the *E. coli* BL21 transformants containing the recombinant pMAL-p2ΔlacI<sup>Q</sup>-*inuJ* plasmids**



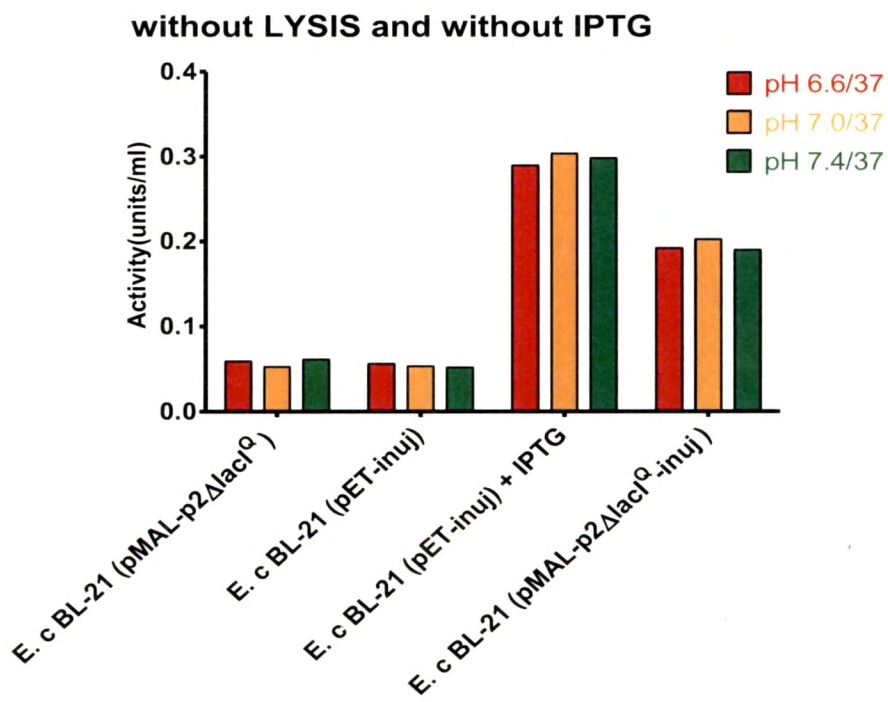
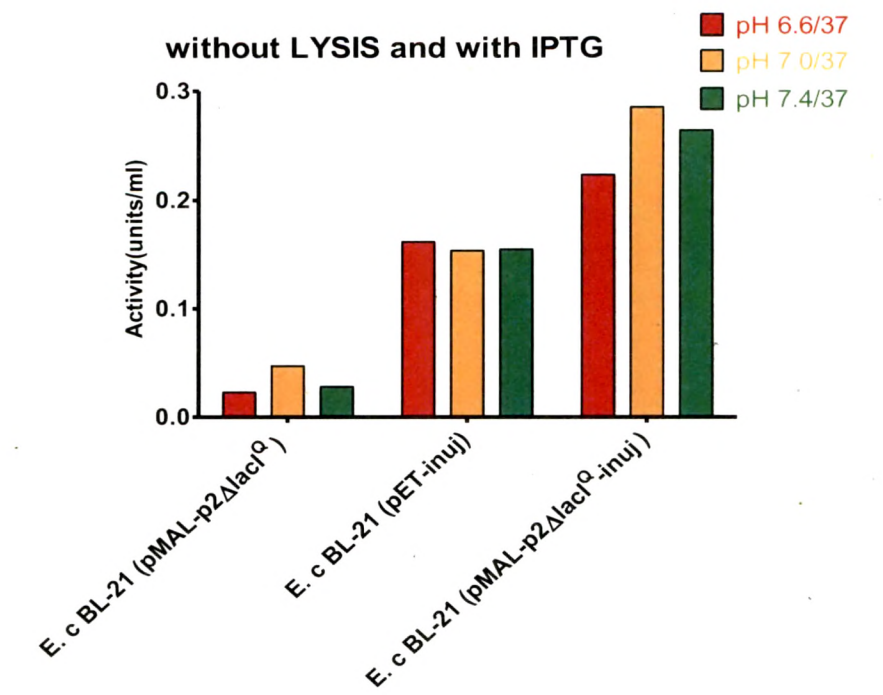
### 5.3.2 Constitutive expression of preiplasmic expression pMAL-p2ΔlacI<sup>Q</sup>-*inuJ*.

Constitutive periplasmic expression of pMAL-p2ΔlacI<sup>Q</sup>-*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<sup>Q</sup>-*inuJ* plasmid clearly demonstrated the disruption of *IacI*<sup>Q</sup> site (Fig. 5.10). The plasmid pMAL-p2ΔlacI<sup>Q</sup>-*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



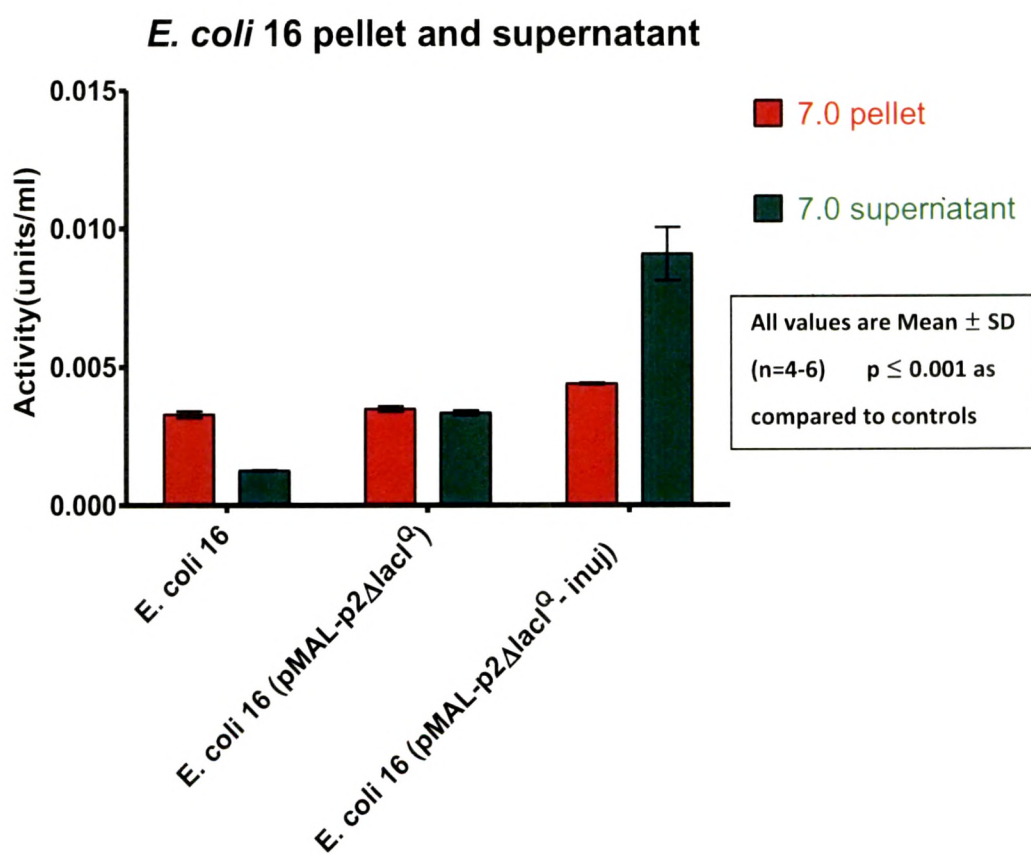


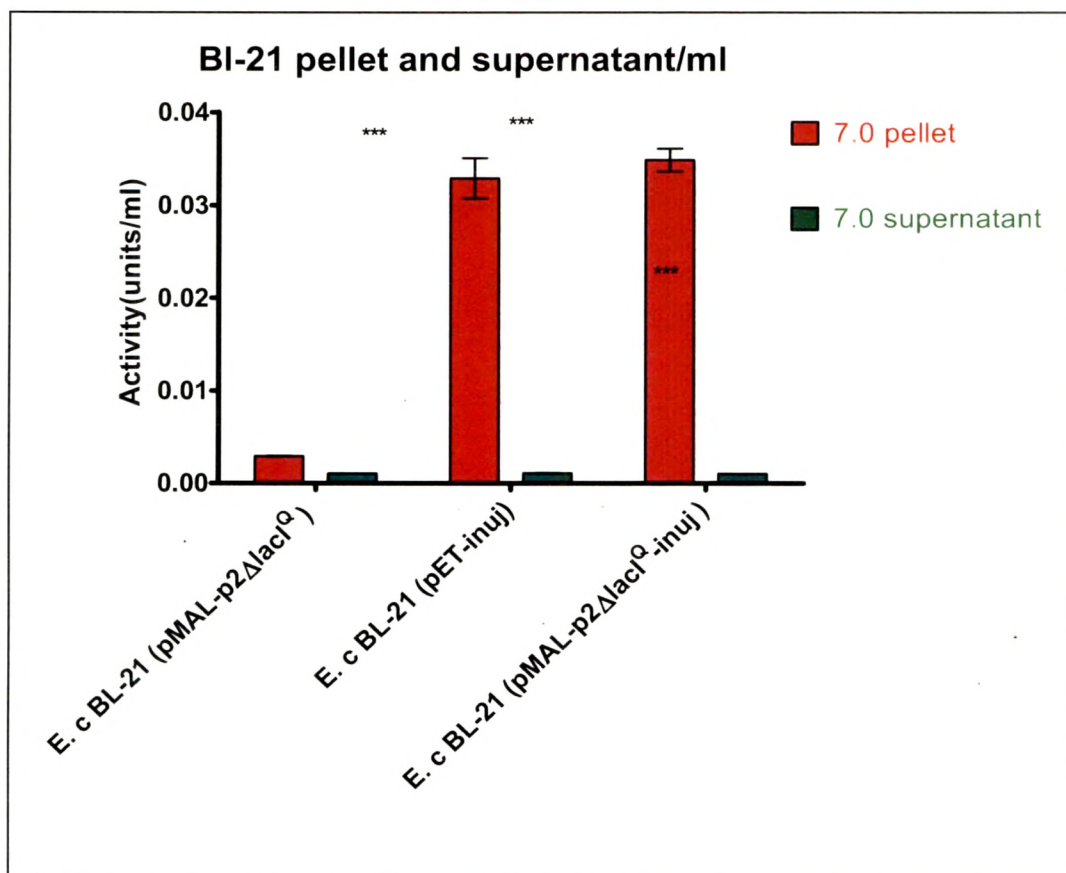


### 5.3.3 Inulosucrase activity in the supernatant of *E. coli* 16 integrants containing pMAL-p2ΔlacI<sup>Q</sup>-*inuJ* plasmid

The enzyme activity in *E. coli* 16 integrants (pMAL-p2ΔlacI<sup>Q</sup>-*inuJ*) was performed in the supernatant along with pellet. *E. coli* 16 integrants (pMAL-p2ΔlacI<sup>Q</sup>-*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<sup>Q</sup>-*inuJ*) to determine its secretion into the medium. Interestingly, the supernatant of *E. coli* BL21 (pMAL-p2ΔlacI<sup>Q</sup>-*inuJ*) did not show any activity but showed activity in the lysate **Fig. 5.11**.

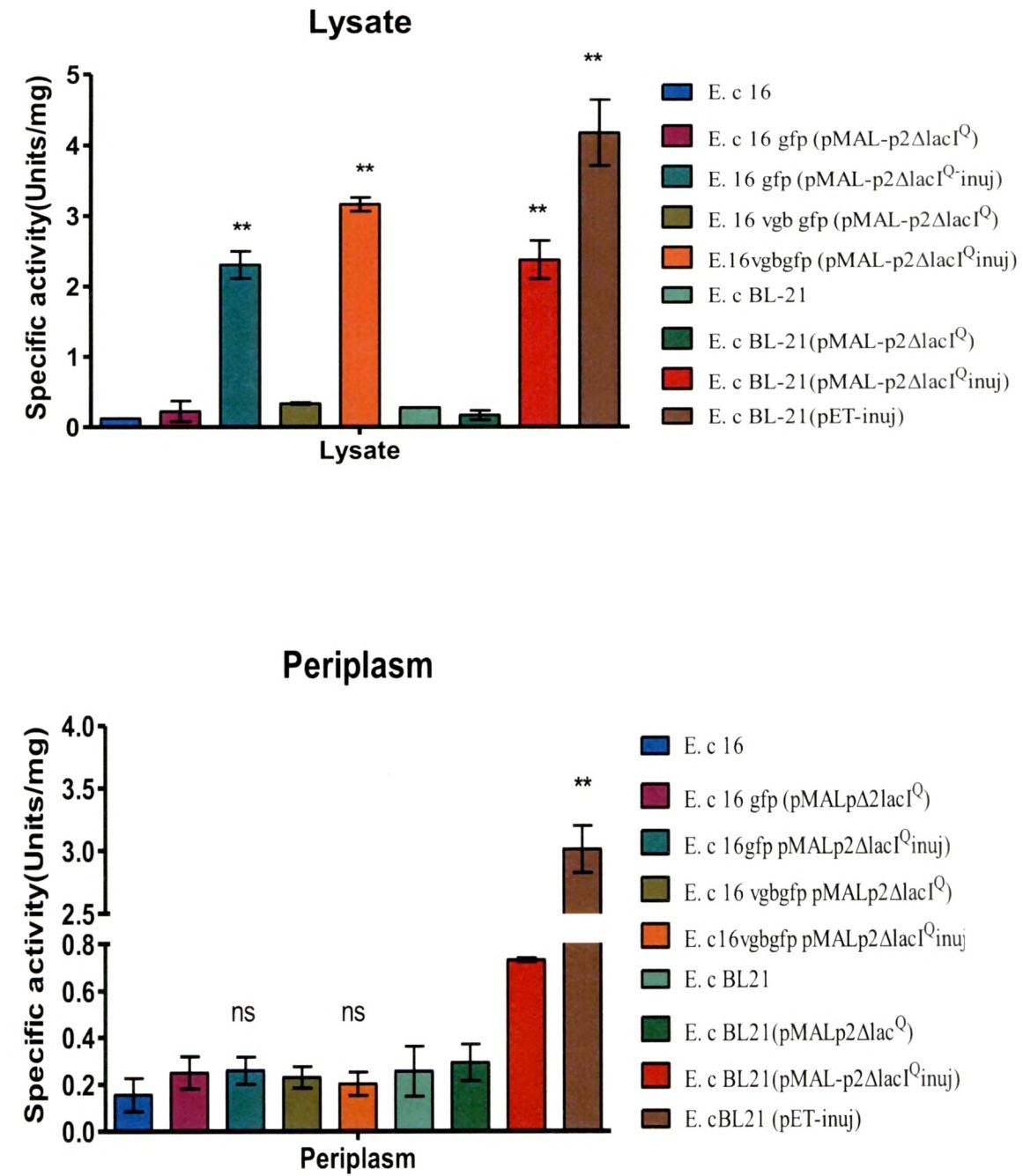
**Fig. 5. 11: Activity of inulosucrase enzyme in *E. coli* 16 integrant and BL21 containing pMAL-p2ΔlacI<sup>Q</sup>-*inuJ***



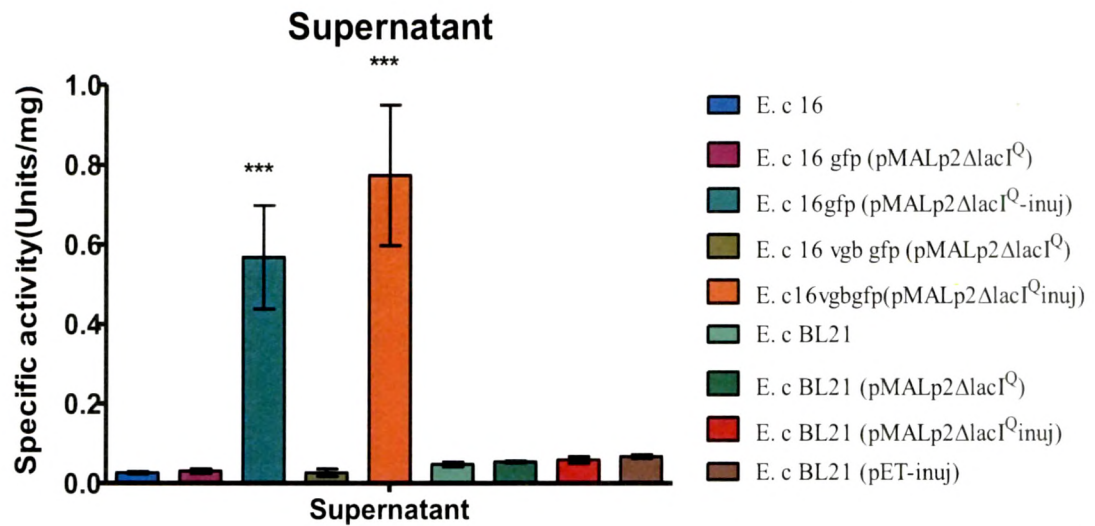


Thus, only the *E. coli* 16 integrants (pMAL-p2ΔlacI<sup>Q</sup>-*inuJ*) transformants showed the protein in the supernatant. Another set of experiment was carried out to check for the presence of the enzyme in the periplasm. Periplasmic proteins were extracted by using the method of Kustu *et al.*, (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<sup>Q</sup>-*inuJ*).



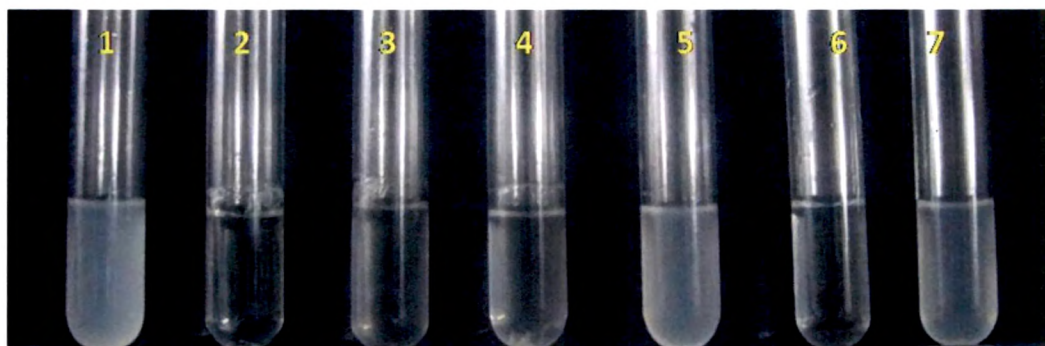




All values are Mean  $\pm$  SD (n=4-6)  $p \leq 0.001$  as compared to controls

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 converts 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-p2ΔlacI<sup>Q</sup>; 5.

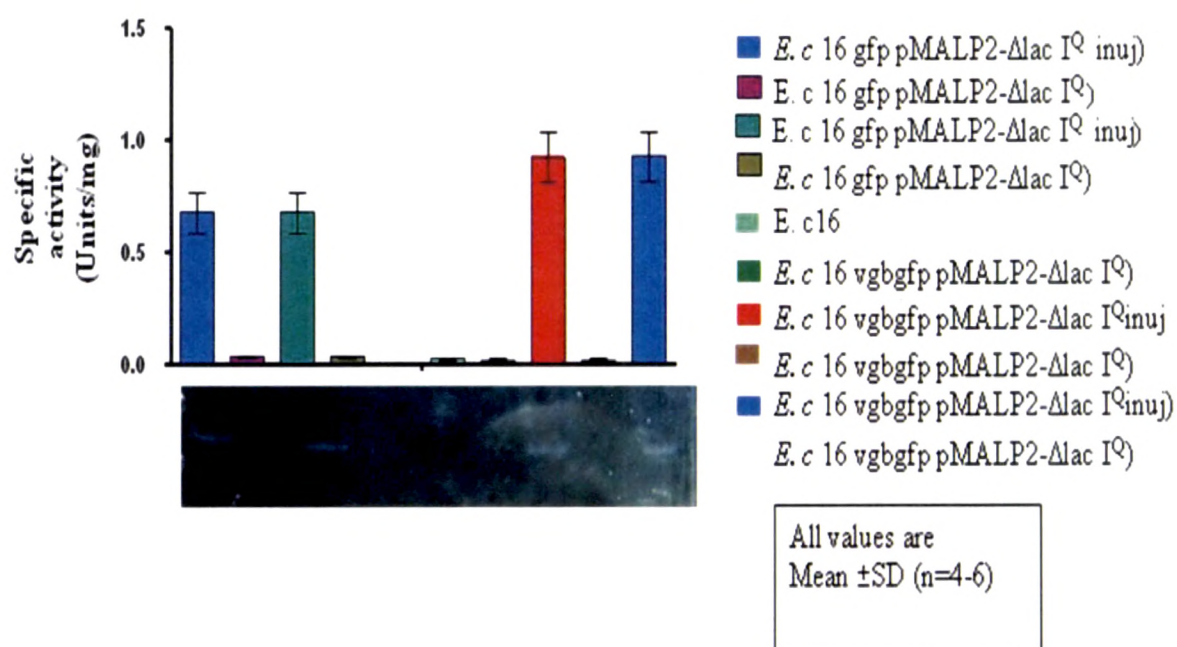
*E. coli* 16 containing pMAL-p2 $\Delta$ lacI<sup>Q</sup> *inuJ*; 6. *E. coli* 16; 7. *E. coli* 16 integrant containing pMAL-p2 $\Delta$ lacI<sup>Q</sup> *inuJ*.

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-p2 $\Delta$ lacI<sup>Q</sup> *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

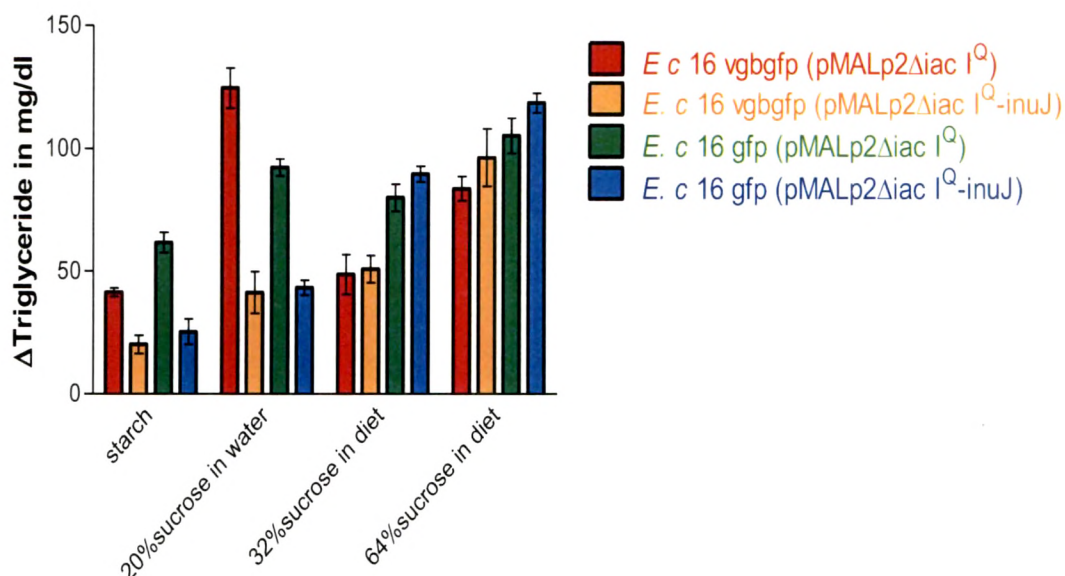
TG level were significant ( $P < 0.01$  value) in 20% sucrose fed groups. In fecal sample, *E. coli* 16 integrant colonies showed fluorescence, antimicrobial activity and ampicillin resistance. After 21 days, approximately  $10^{5-7}$  colonies of *E. coli* 16 integrants were present in per gram of fecal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

**Table 5.7. Bacterial isolates count in fecal samples of rats.**

	<i>E. coli</i> 16vgbgfp pMALp2ΔlacI <sup>Q</sup>	<i>E. coli</i> 16vgbgfp pMALp2ΔlacI <sup>Q</sup> - inuj	<i>E. coli</i> 16gfp pMALp2ΔlacI <sup>Q</sup>	<i>E. coli</i> 16gfp pMALp2ΔlacI <sup>Q</sup> - in
Starch	$4.50 \times 10^7$	$1.15 \times 10^7$	$8.90 \times 10^7$	$4.40 \times 10^6$
20%Sucros	$3.72 \times 10^7$	$9.10 \times 10^7$	$6.60 \times 10^7$	$4.95 \times 10^6$
32%Sucros	$6.50 \times 10^6$	$2.85 \times 10^6$	$8.50 \times 10^6$	$1.25 \times 10^5$
64%Sucros	$1.20 \times 10^7$	$1.07 \times 10^7$	$5.25 \times 10^7$	$3.40 \times 10^6$



**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.

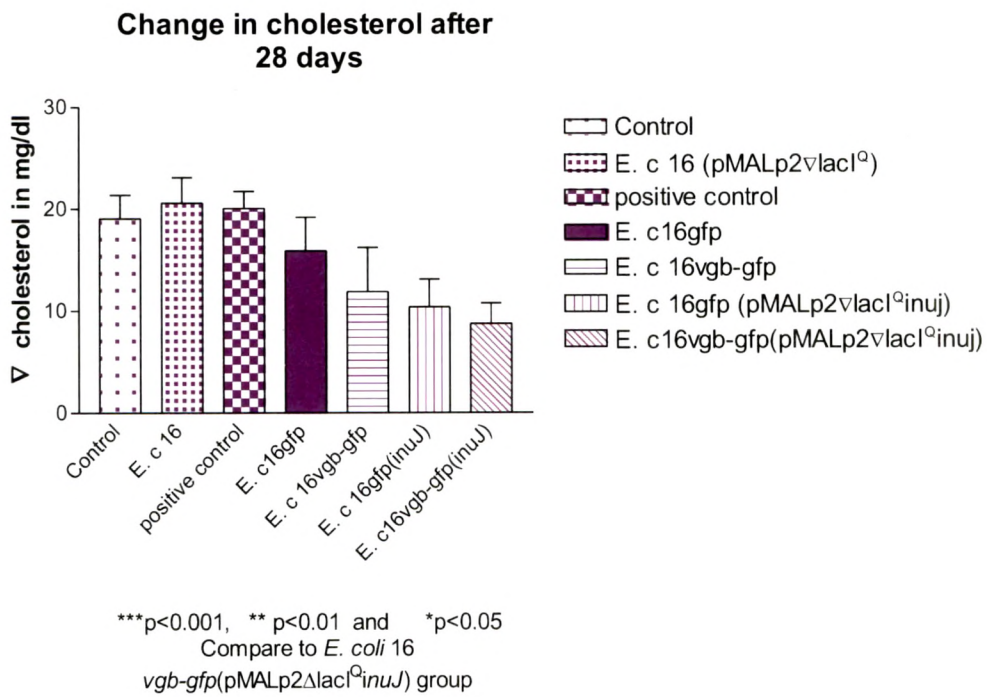
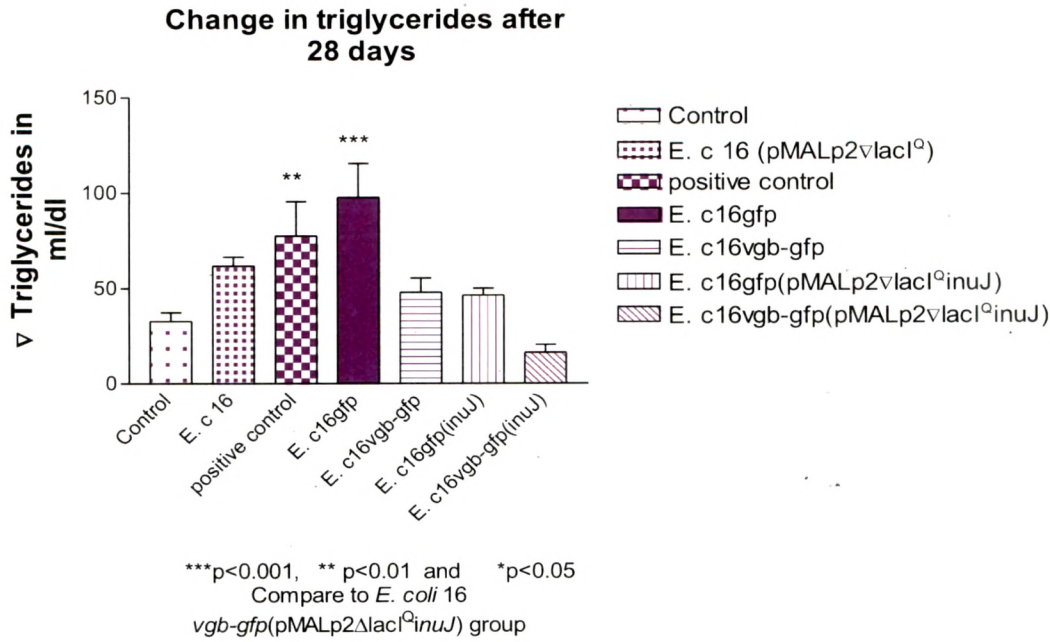
### 5.3.6. Short term effects (28 days) of probiotic harbouring *inuJ* gene.

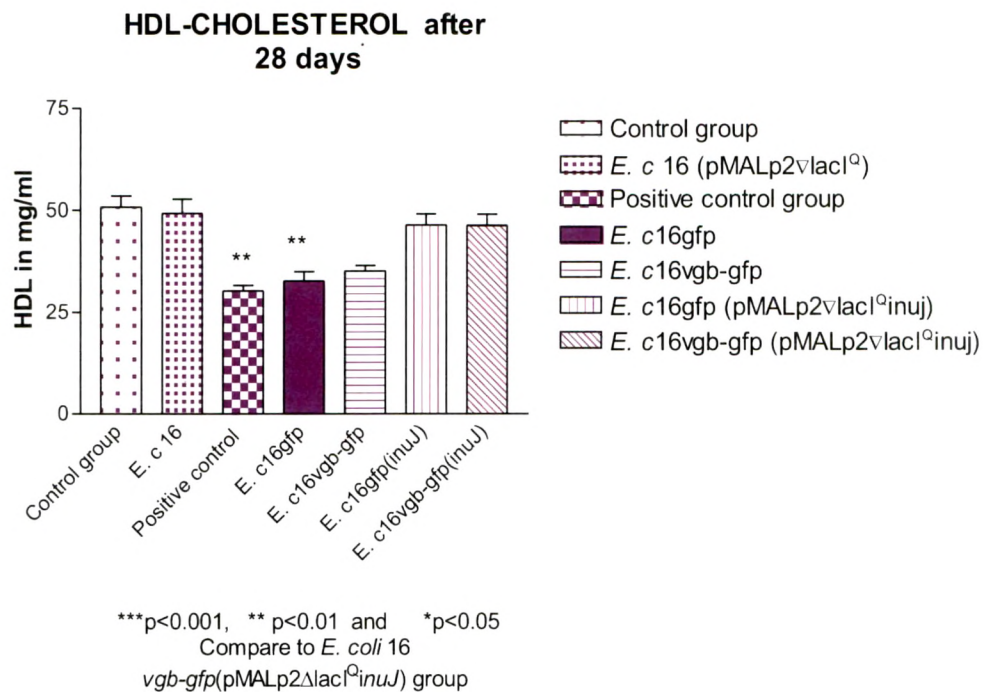
#### 5.3.6.1. Serum Lipid profile.

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. coli* 16 vgbgfp (pMALp2Δ*lacI*<sup>Q</sup>*inuJ*)) **Fig.5.16** . No Significant change was observed in TG of 20% sucrose fed positive control groups and *E. coli* 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

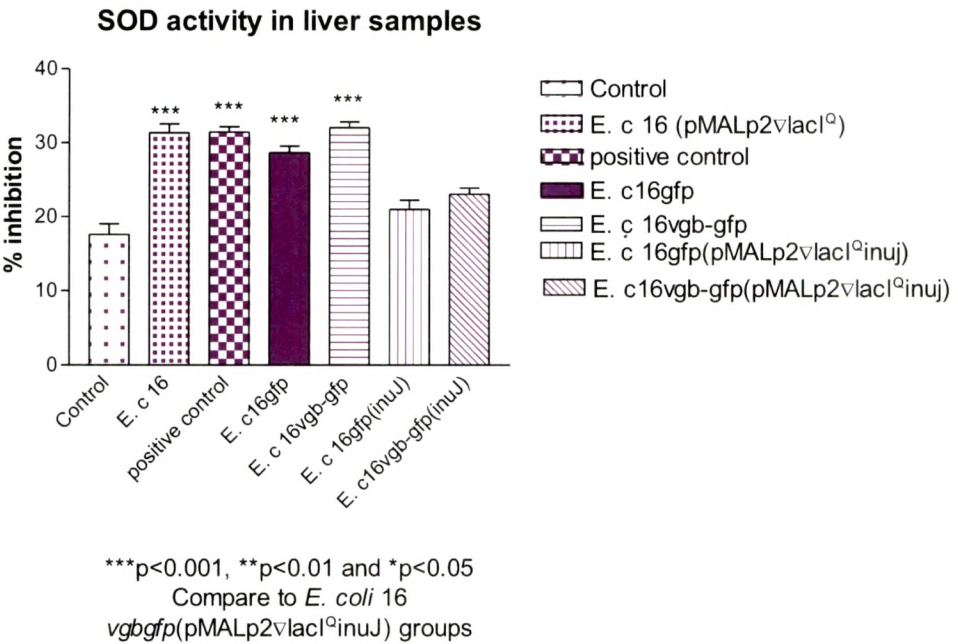
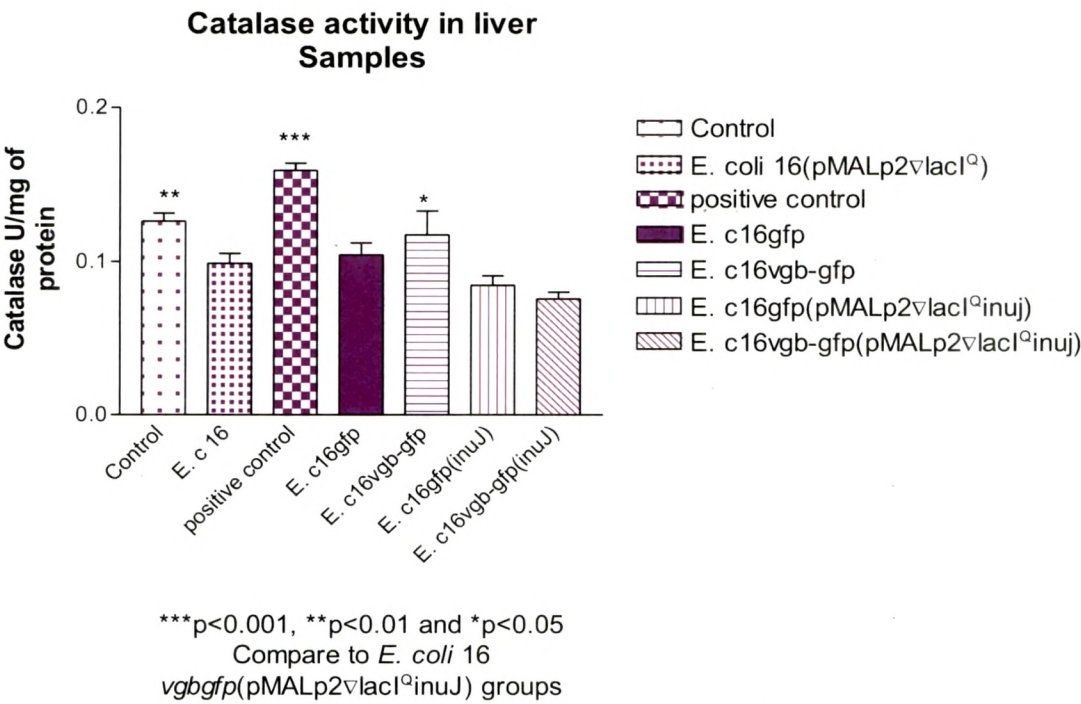


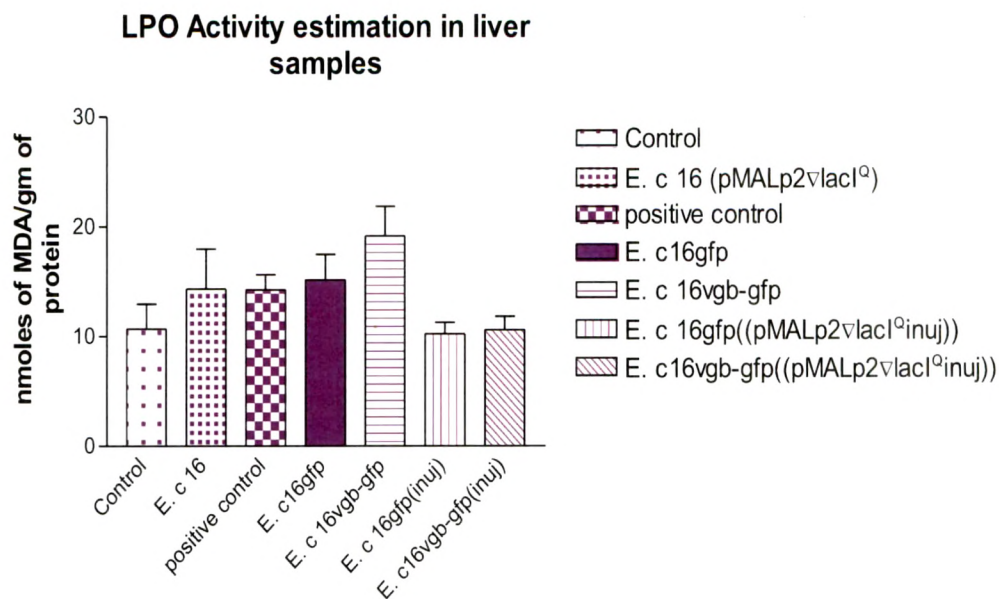


### 5.3.6.2 Antioxidant status of liver

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*<sup>Q</sup>-*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*<sup>Q</sup>-*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





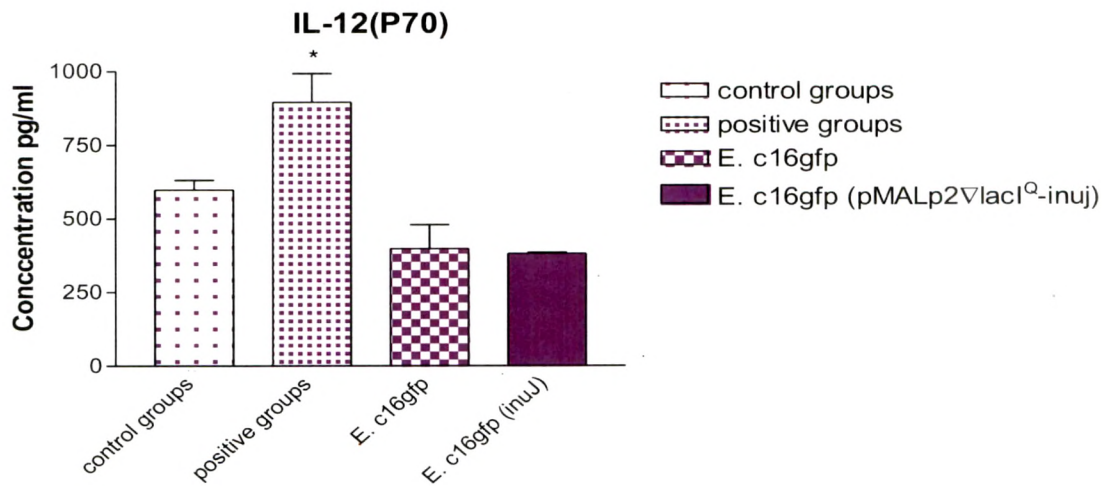
\*\*\*p<0.001, \*\*p<0.01 and \*p<0.05  
Compare to *E. coli* 16  
*vgbgfp*(pMALp2v-lacI<sup>Q</sup>*inuJ*) groups

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

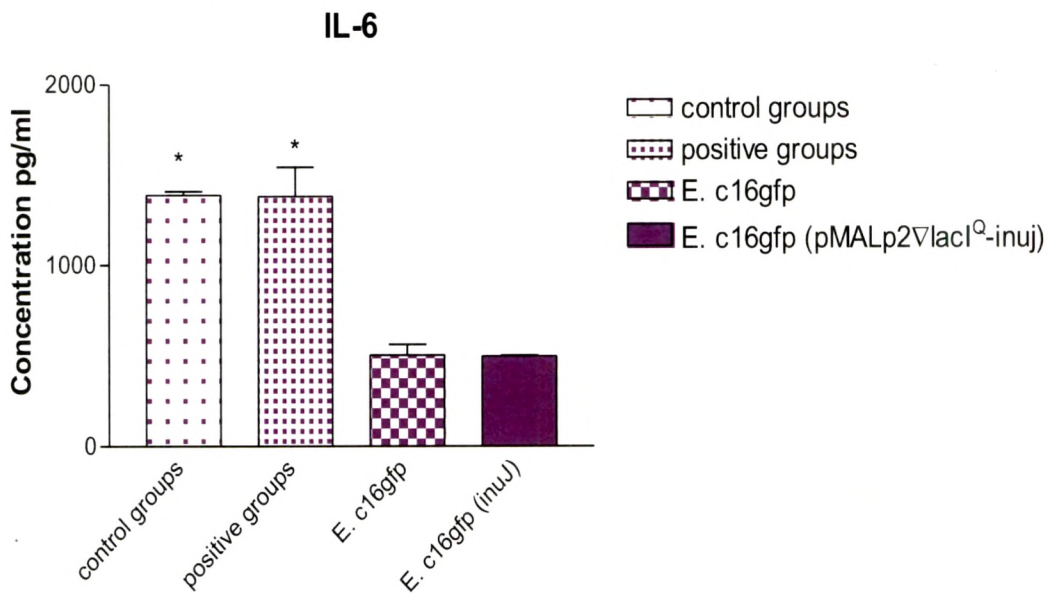
Pro-inflammatory cytokines like IL-12, IL-6, IFN $\gamma$ , IP-10 and Rantes were estimated in selected groups. The levels of IL-12 and IFN $\gamma$  were significantly increased in positive control groups compared to other groups such as control, *E. c 16gfp* and *E. c 16gfp (inuJ)* groups ( $p < 0.05$ ) 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**

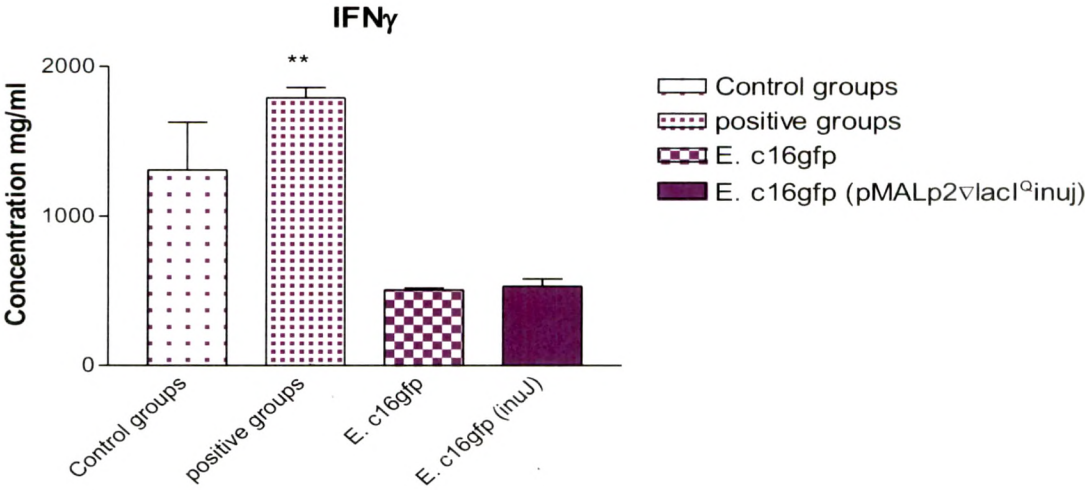


\*\*\*p<0.001, \*\*p<0.01 and \*p<0.05  
Compare to *E. coli* 16  
*gfp*(pMALp2∇lacI<sup>Q</sup>inuJ) groups

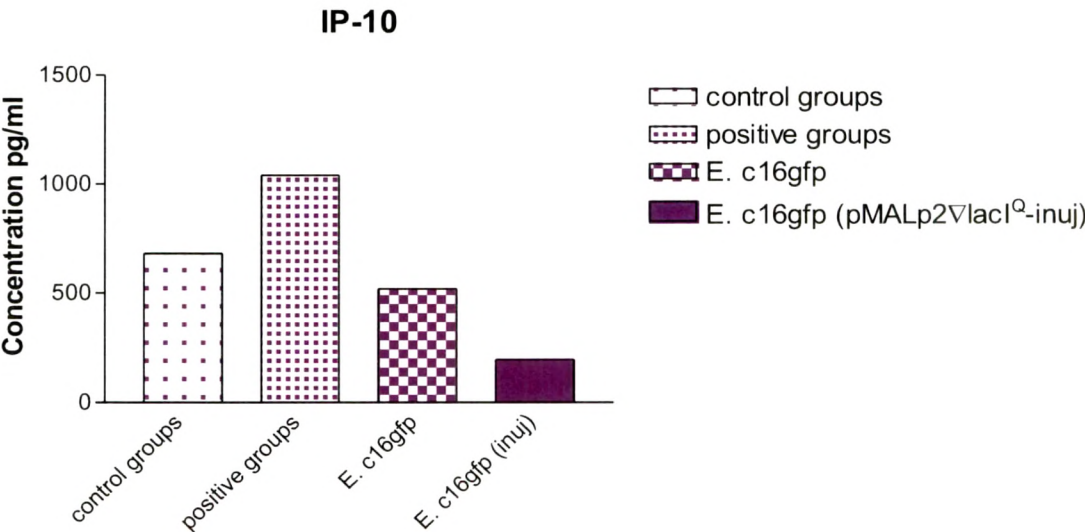


\*\*\*p<0.001, \*\*p<0.01 and \*p<0.05  
Compare to *E. coli* 16  
*gfp*(pMALp2∇lacI<sup>Q</sup>inuJ) groups

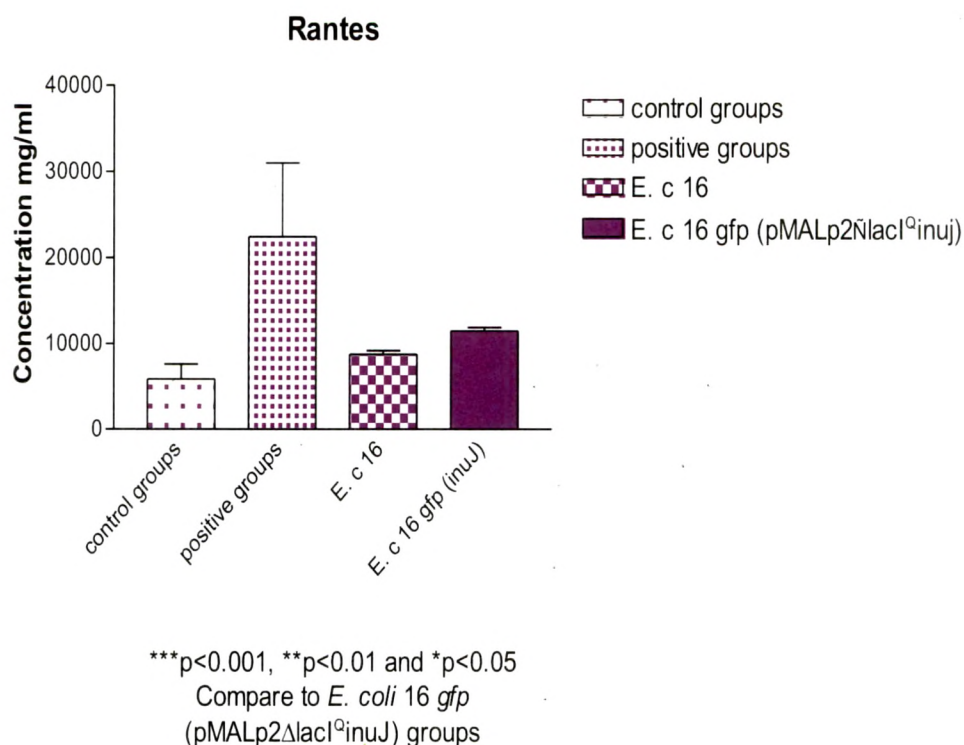
Chapter 5: Effect of probiotic *E. coli* 16 strain containing inulosucrase gene in alleviation of sucrose mediated metabolic disorder



\*\*\*p<0.001, \*\*p<0.01 and \*p<0.05  
Compare to *E. coli* 16  
gfp(pMALp2 $\nabla$ lacI<sup>Q</sup>inuJ) groups



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



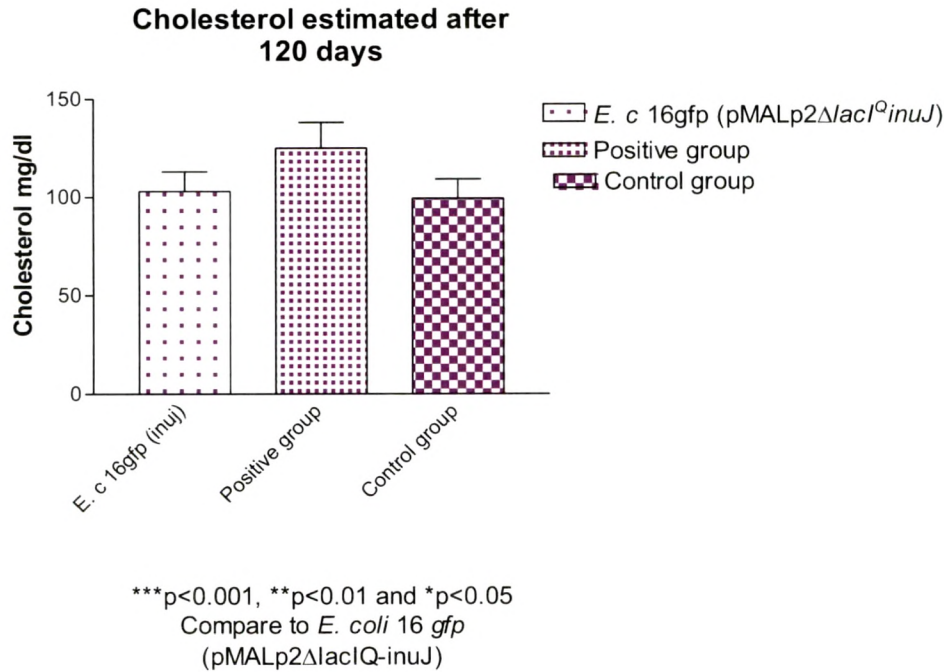
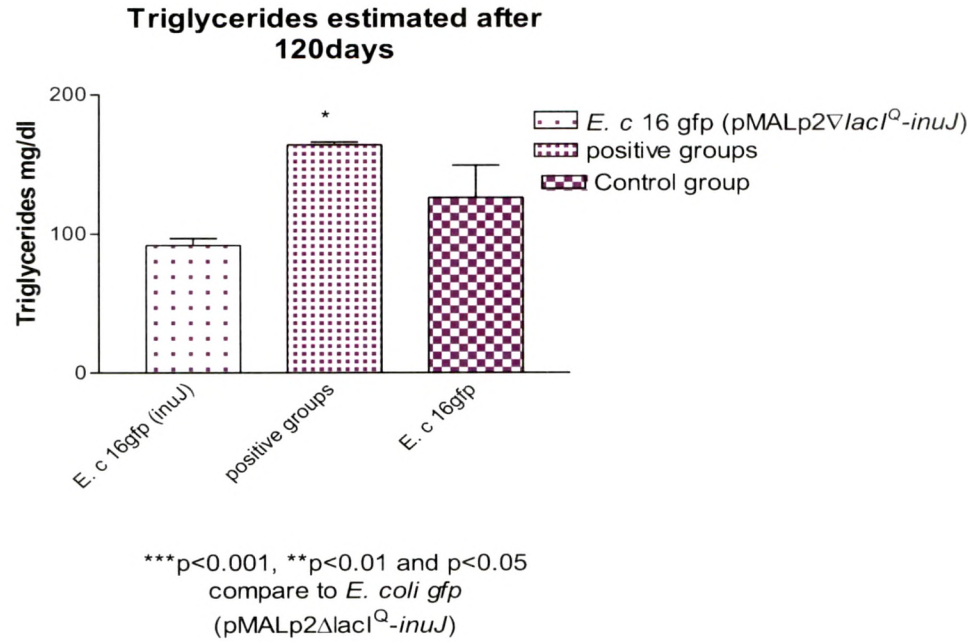
**5.3.7 Long term effects (120 days) of probiotic harbouring *inuJ* gene.** Long term effects of 20% sucrose in drinking water were monitored in three rat groups as mentioned in section 5.2.7.5.

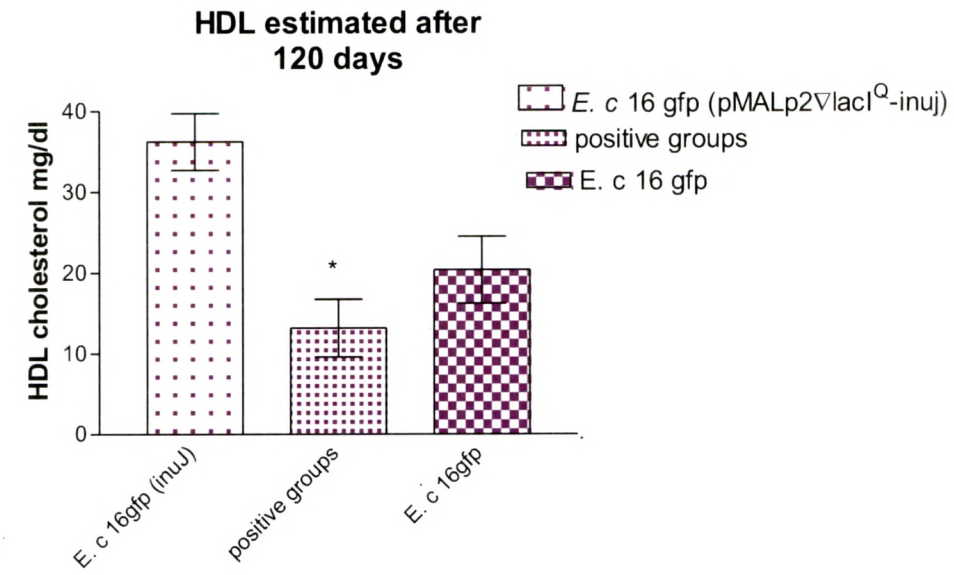
#### 5.3.7.1 Biochemical analysis of serum samples.

**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**).

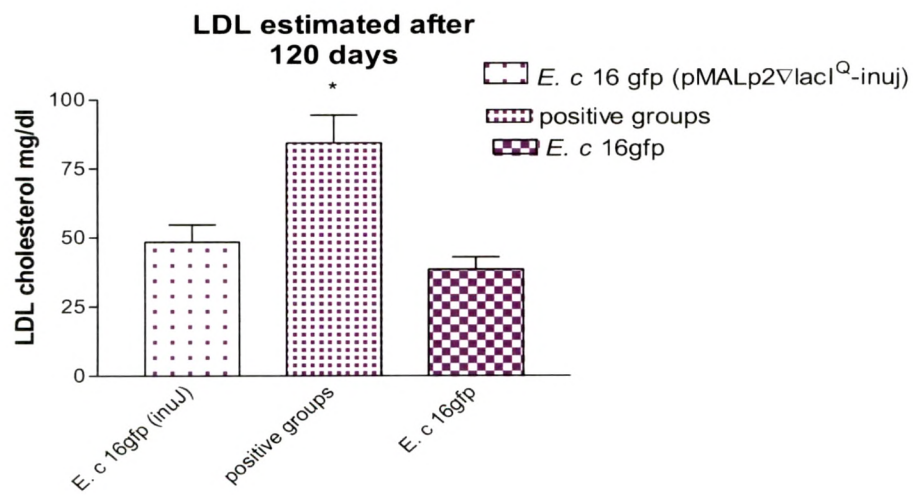


Fig. 5.19. Serum lipid profiles in rats fed sucrose diet for 120 days





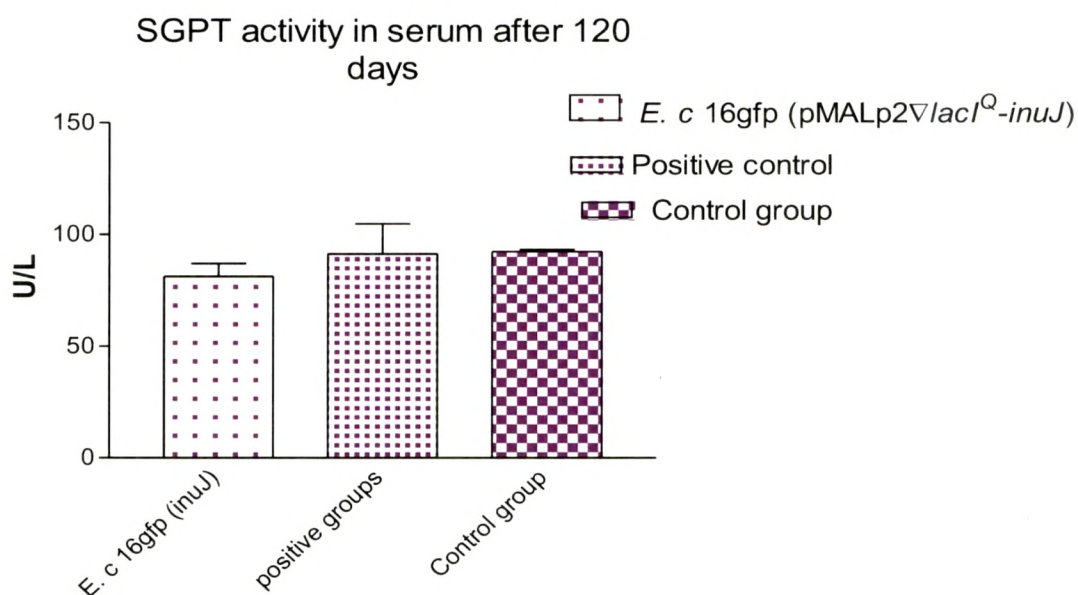
\*\*\* $p < 0.001$ , \*\* $p < 0.01$  and \* $p < 0.05$   
Compare to *E. coli* 16 *gfp*  
(pMALp2ΔlacIQ-inuJ)

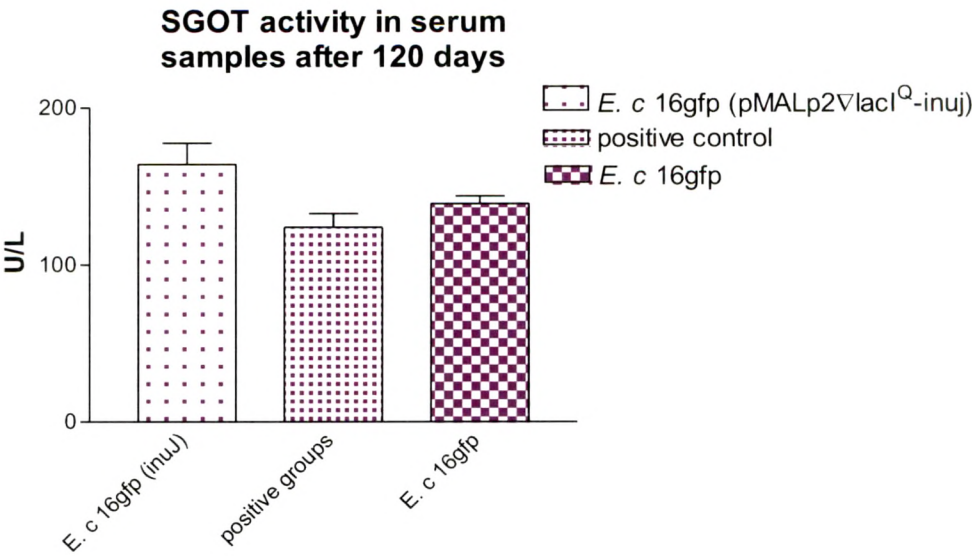


\*\*\* $p < 0.001$ , \*\* $p < 0.01$  and \* $p < 0.05$   
Compare to *E. coli* 16 *gfp*  
(pMALp2ΔlacIQ-inuJ)

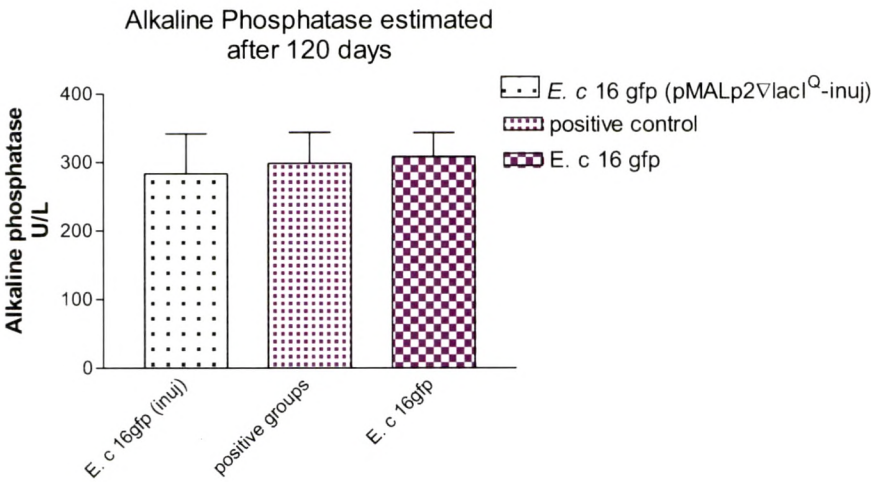
B) Liver function test. No significant changes were observed in SGPT, SGOT, Alkaline phosphatase activity, total protein, albumin and globulin in all the three groups (Fig. 5.20). Total, direct and indirect bilirubin was same in both *E. coli* 16 gfp and *E. coli* 16 gfp (pMALp2ΔlacI<sup>Q</sup>-inuJ)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

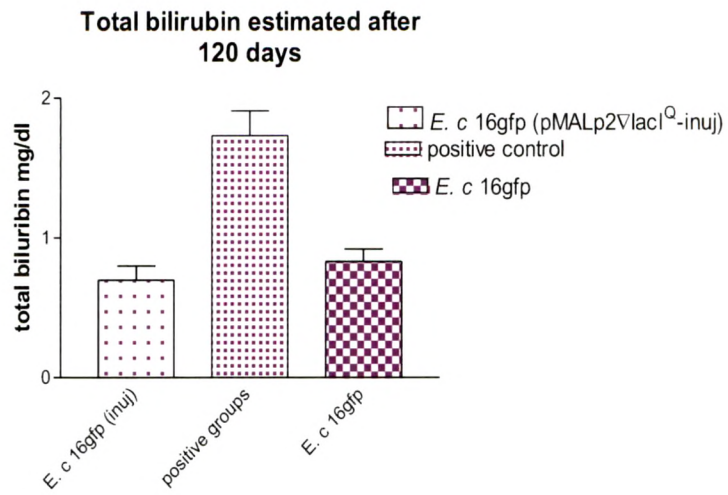
**Fig. 5.20. Liver function tests in rats fed sucrose diet for 120 days**



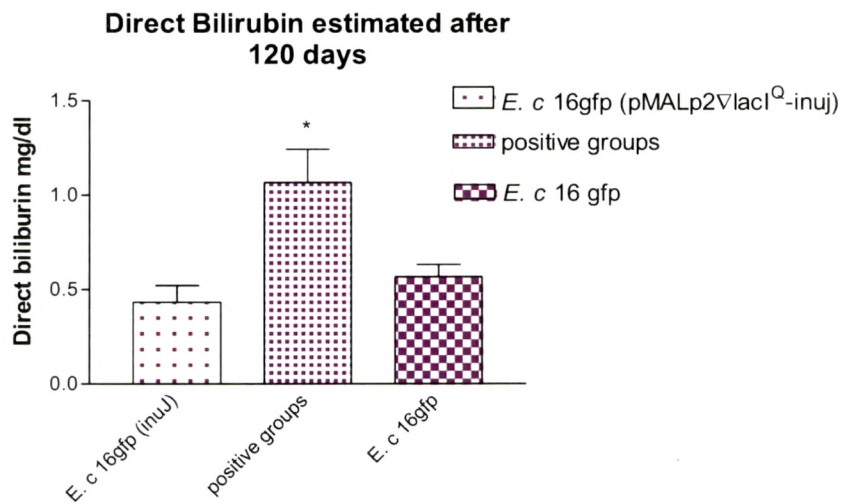


\*\*\*p<0.001, \*\*p<0.01 and \*p<0.05  
Compare to *E. coli* 16 *gfp*  
(pMALp2ΔlacIQ-inuJ)

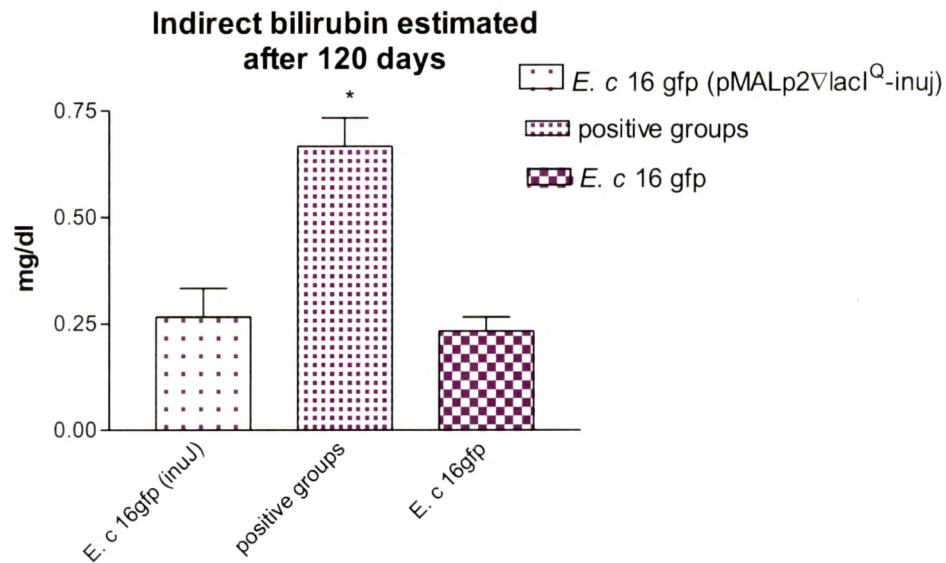




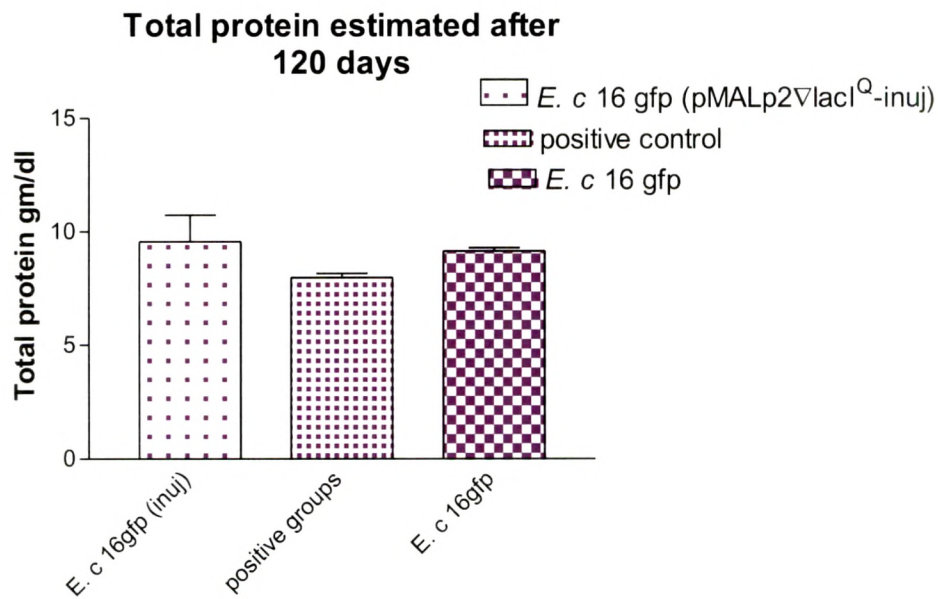
\*\*\* $p < 0.001$ , \*\*\* $p < 0.01$  and  $p < 0.05$   
Compare to *E. coli* 16gfp  
(pMALp2 $\Delta$ lacI<sup>Q</sup>-inuJ)



\*\*\* $p < 0.001$ , \*\* $p < 0.01$  and \* $p < 0.05$   
Compare to *E. coli* 16gfp  
(pMALp2 $\Delta$ lacI<sup>Q</sup>-inuJ)

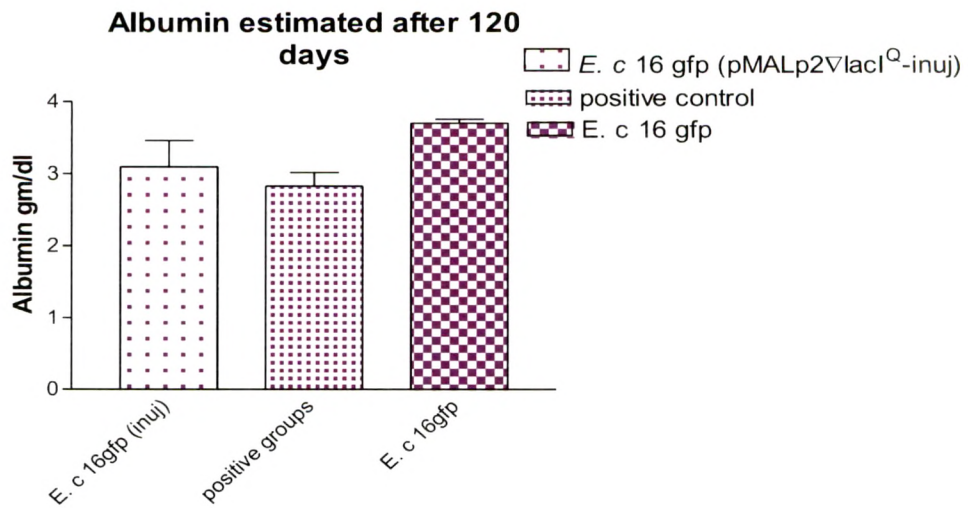


\*\*\*p<0.001, \*\*p<0.01 and \*p<0.05  
Compare to *E. coli* 16 gfp  
(pMALp2 $\Delta$ lacI<sup>Q</sup>-inuJ)

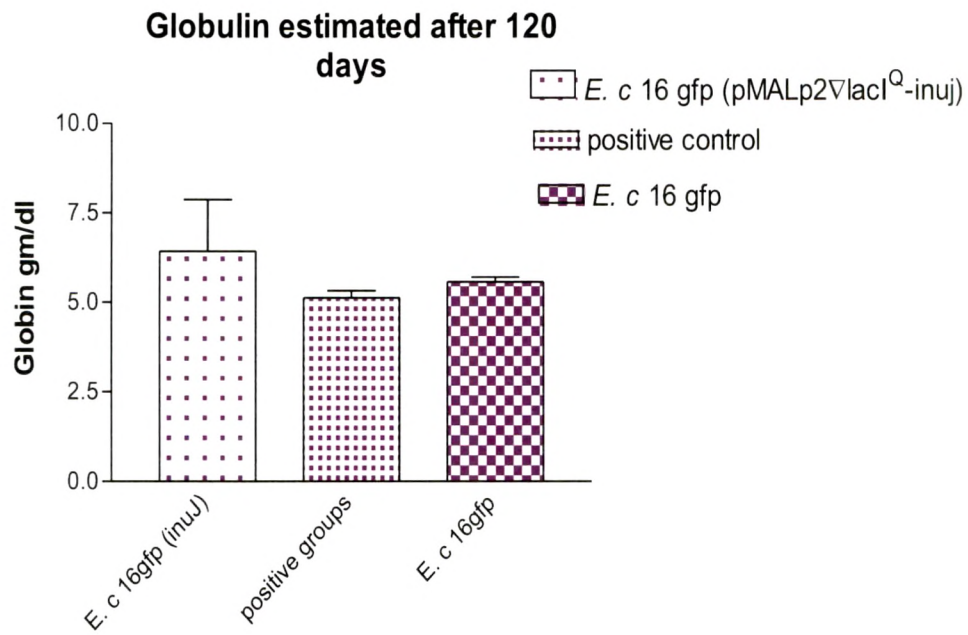


\*\*\*p<0.001, \*\*p<0.01 and \*p<0.05  
Compare to *E. coli* 16 gfp  
(pMALp2 $\Delta$ lacI<sup>Q</sup>-inuJ)





\*\*\* $p < 0.001$ , \*\* $p < 0.01$  and \* $p < 0.05$   
Compare to *E. coli* 16 *gfp*  
(pMALp2 $\Delta$ lacIQ-inuJ)



\*\*\* $p < 0.001$ , \*\* $p < 0.01$  and \* $p < 0.05$   
Compare to *E. coli* 16 *gfp*  
(pMALp2 $\Delta$ lacIQ-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. coli* 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.

*Chapter 5: Effect of probiotic E. coli 16 strain containing inulosucrase gene in alleviation of sucrose mediated metabolic disorder*

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 secrete 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.