Chapter 3:

Determining the effect of fructose and prebiotic produced by *Escherichia coli* Nissle 1917 harboring *glucose facilitator protein* and *mannitol dehydrogenase* genes on iron absorption.

3.1 Introduction

Iron deficiency and low iron status is the most common micronutrient deficiency all around the world (Bailey *et al*., 2015), and women of reproductive age are a susceptible population since they have high iron requirements (Hallberg *et al*., 1991; 1995). Various components present in diet that enhance iron absorption include ascorbic acid and meat and those that inhibit absorption include calcium, polyphenols and phytates (Hallberg *et al*., 2000).

Sugars increase the liver deposits of iron by enhancing iron bioavailability as they reduce iron into Fe^{+2} states or chelate inorganic iron and form stable soluble sugar-iron complexes which are readily absorbed across the intestinal mucosa (Tsuchiya *et al*., 2013).Fructose significantly increases iron-induced ferritin formation in human intestinal cells (Christedes *et al*., 2013). In Western diets, the major source of fructose in the diet is not fruit, but sucrose and high-fructose corn syrup, and in particular HFCS-55, consequently fructose levels in both the gut and portal vein may be elevated (White, 2008; Marriott *et al*., 2010; Ogden *et al*., 2011;Whitton *et al*., 2011). The amount of HFCS-55 present in sweetened beverages increase iron bioavailability similar to that of fructose.

The absorption of non-haem iron is enhanced by lactic acid-fermented foods in humans probably because of their ability to activate phytases and lower pH due to organic acids production (Gillooly *et al*., 1982; Brune *et al*., 1992; Goossens *et al*., 2005; Scheers *et al*., 2016). Additionally, probiotic strain *Lactobacillus plantarum* 299v and *Lactobacillus acidophilus* supplementation improved iron absorption thereby increasing hemoglobin (Hb) levels (Costa *et al., 2*000; Hoppe *et al*., 2015). Fermentation of almond milk with potentially probiotic bacteria enhanced the uptake of iron by Caco-2 cells, specifically with either *B. bifidum* or *B. longum* and *L. rhamnosus* (Bernat *et al*., 2015).

Prebiotics including fuctooligosachharide, inulin, mannitol, gluconic acid also have an iron absorption increasing property (Yeung *et al*., 2005). These prebiotics in the gastrointestinal tract are utilized by the gut microbiota and are converted into short chain fatty acids (SCFA) also help in iron absorption because they lower the pH of colon and

thus, help in absorption by releasing iron from complexes (Conesa *et al.,* 2007). Consumption of *Bifidobacterium lactis* HN019 and prebiotic fortified milk for a year increased weight gain and improved iron-deficiency in preschoolers of Indian population (Sazawal *et al*., 2010). *Lactobacillus* strains degrade phytic acid by inherent phytases during fermentation where by the bound iron is made available for absorption (Fischer *et al*., 2014).

Fructose when is taken up by *Ec*N*(pqq-glf-mtl*K*)* with the help of glucose facilitator protein, produces two prebiotics, gluconic acid and mannitol by the action of PQQ dependent glucose dehydrogenase and mannitol dehydrogense enzyme, respectively (Somabhai *et al.*, 2016). These prebiotics are converted to SCFA which lower the pH of the colon which in turn helps to reduce ferric ions into ferrous ions enhancing iron absorption. Also, *Ec*N *(pqq-glf-mtl*K*)* probiotic has been shown to ameliorate the metabolic syndrome caused by consumption of excess fructose. Hence, the present study investigates the potential of *Ec*N *(pqq-glf-mtl*K*)* in facilitating iron absorption and ameliorating metabolic syndrome caused by fructose consumption.

3.2 Materials and methods:

3.2.1 Plasmid, Bacterial strains and culture condition

*Ec*N was obtained from Dr.Ulrich Sonnenborn (Ardeypharm GmbH, Loerfeldstrabe 20, Herdecke, Germany) as a generous gift. All plasmid constructs and bacterial strains used in the present study are summarized in Table 3. Routine DNA manipulations were done in *E. coli* DH10B (Invitrogen, USA) using standard molecular biology protocols from Sambrook and Russell (2001). For probiotics supplementation to different rat groups, overnight grown culture of different strains in Luria Broth (LB) at 37 ^{o}C was re-inoculated in fresh L.B tubes to achieve final colony forming unit (CFU) of 10^9 per ml. One ml of fresh culture (CFU of 10^9/ml) was taken from the tube, centrifuged and washed twice with normal saline before oral administration to rats. Iron was given in the ferric citrate form in the diet, ascorbic acid will also be given mixed in the diet and 20% fructose will be given in drinking water.

Plasmid / strains	Characteristics	Reference
Plasmids		
pJET vector	Cloning vector, PlacUV5, rep (pMB1), T7 promoter, Apr	ThermoScientific CloneJET PCR Cloning Kit
pAN1	pJET vector containing p*tac*-pqq*, Apr	This study
pAN6	pJET vector containing p*tac*-pqq-glf-mtlK*, Apr	This study

Bacteria		
E.coli DH10B	Host strain for routine DNA manipulation.	(Invitrogen , USA)
*Ec*N-2	*Ec*N containing vgb and gfp genes in the genome.	Singh *et al.*, 2014
*Ec*N(pAN1)	*Ec*N-2 containing *Ec*N (pAN1).	This study
*Ec*N(pAN6)	*Ec*N-2 containing *Ec*N (pAN6).	This study

Table 3.1: Plasmid and bacterial strains used in the study

3.2.2 Animals

Adult albino male Charles Foster rats (180-200 g) were used for animal studies. All rats were maintained in controlled condition as per committee guidelines *i.e.* temperature (25 ± 1 ºC), relative humidity (45.5 %) and photoperiod cycle (12 h light: 12 h dark)). Free access to food and water was provided as per recommended by committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines of animal ethical committee of M. S. University of Baroda, India, Registration number 938/a/06/CPCSEA.

3.2.3 Experimental design

For present study, Charles Foster rats were divided initially into two different groups as follows: Iron deficient group: AIN-93 iron depleted diet was fed to the rats for 10 weeks (n=60) and the control group (n=6) was fed with normal diet. Composition of the diet is shown in Table 3.2.

For confirmation of iron deficiency, iron levels in the blood were checked. After confirmation of iron deficiency, probiotics (10^9 *cfu*) was supplemented to all groups except Group 1 and 2 once per week till one month as per Singh *et al.*, 2014. The fecal plate count was done for confirmation of colonization of the probiotic. The iron deficient group was then divided into 10 groups (n=6) and different treatments were given for 4 weeks as follows: Group 1 received normal diet. Group 2 received Iron deficient diet. Group 3 received 20% fructose. Group 4 received Fructose + iron. Group 5 received Iron

+ Ascorbic Acid (150 mg/kg diet) .Group 6 received Iron+*Ec*N-2. Group 7 received Fructose+Iron+*Ec*N-2. Group 8 received Iron +*Ec*N (pAN1).Group 9 received Fructose +Iron +*Ec*N(pAN1).Group 10 received Iron+ *Ec*N (pAN6). Group 11 received Fructose + Iron + *Ec*N (pAN6). All the groups were given iron containing diet except Group 3.

	Normal Diet (g/kg)	Iron deficient Diet(g/kg)
Casein	200	200
L-Cystine	3.000	3.000
Corn Starch	397.486	397.486
Fructose	30	30
Soybean Oil	70.000	70.000
Mineral Mix	35.000	35.000
Ferric citrate	.212	000
Vitamin mix	10.0	10.0
Choline bitartrate	2.5	2.5

Table 3.2: Composition of AIN-93 iron depleted diet and normal diet. (Saini *et al*., 2014)

Probiotics (10^9 *cfu*) was supplemented to all groups except Group 1 and 2 once per week till one month as per Singh *et al*,.2014. Iron was given in the form of ferric citrate (o.2g) in the diet, 20% fructose will be given in the drinking water and the amount of ascorbic acid to be given is 150 mg/kg of diet. At the end of the10weeks, iron-depleted and control rats were fasted overnight and euthanized under ether anesthesia.

Livers were dissected and then rinsed with Diethyl pyrocarbonate-treated water. Approximately 1 g of liver section was frozen in liquid nitrogen in 15 mL RNase-free falcon tubes therby immediately stored at −80°C for RNA extraction. Serum clinical enzymes were analysed.

3.2.4 Preparation of cell lysate and tissue homogenates

Blood was collected by orbital sinus puncture in EDTA coated and normal tubes followed by centrifugation at 1500 g for 10 minutes. Plasma and serum was collected

separately in fresh tubes and stored in -80 ^{O}C till use. Pack cell volume (PCV) was washed thrice with normal saline prior to lysis in ice cold water. Cell lysate so obtained was centrifuged at 15,000 g for 10 min and fresh supernatant was used for enzyme assays. Liver was collected and washed with PBS immediately after sacrificing rats. Liver homogenates were prepared in different buffers for antioxidant enzyme activity.

3.2.5 Biochemical assays

Superoxide dismutase (SOD) activity was measured by a method which is based on auto-oxidation of pyrogallol monitored spectrophotometrically at 420 nm (Marklund *et al*., 1974). Catalase (CAT) activity was monitored by measuring rate of disappearance of hydrogen peroxide (H_2O_2) spectrophotometer at 240 nm (Beers *et al.*, 1952). SOD and CAT activities are reported as units/mg protein. Reduced glutathione (GSH) was performed as described in Beutler *et al.*, 1963. Lipid peroxidation was estimated by measuring levels of MDA at 412 nm as described in Buege *et al*, 1978.

3.2.6 Liver enzyme test, kidney function test, lipid and iron estimation.

Aspartate transaminase (AST), Alanine transaminase (ALT), triglyceride and total cholesterol in blood plasma were measured using kits as per manufacturer protocol (Beacon Diagnostics Pvt. Ltd. Navsari, India). Iron content from serum were measured using Ferrozine method using kit as per manufacturer protocol (Coral Clinical Systems, Goa, India)

3.2.7 mRNA expression and qRT-PCR

RNA was extracted with Trizol (Invitrogen BioServices India Pvt. Ltd., Bangalore, India) and cDNAs were generated from 1 µg total RNA (Reverse Transcription Kit; Applied Biosystems) following the manufacturer's guidelines. Primers selected for Ferritin-H (FH) and Transferrin (TF) gene were CTTTGCAACTTCGTCGCTCC (forward) and GTCCTGGTGGTAGTTCTGGC (reverse), and GATGAGTGGAGCGTCACGAG (forward) and CTGGCCTGCTATGTAGGCAT (reverse), respectively. PCR was performed by ABI Quant-Studio TM 12K flex Real Time PCR system coupled with SYBR Green

technology (Applied Biosystems) and following cycling parameters. The linearity of the dissociation curve was analysed using the software provided with the thermocycler (QuantStudioTM). Each sample was analyzed in duplicate. The mean cycle time of the linear part of the curve was designated Ct.

3.2.8 Histopathological changes

Liver tissue was fixed with 10 % buffered formalin. Histological sections were stained with hematoxylin and eosin. Pathologist unaware with experimental codes was consulted for analysis.

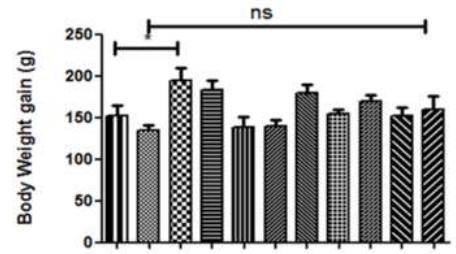
3.2.9 Statistical analysis

All values are expressed as means ± SEM. Differences in lipid peroxidation and antioxidant enzymes (SOD, CAT and GSH) among six different groups were evaluated using the one-way ANOVA followed by Bonferroni comparisons. Differences were considered significant at $P<0.05$. Calculations were done using commercially available statistical software packages (Graph Pad PRISM Version 5.0 La Jolla, CA 92037 USA).

3.3 Results:

3.3.1 Body weight

There was no significant change in the iron deficient control, but as fructose consumption is related to weight gain, similar result was obtained. The rats in fructose control group showed an increased body weight as compared to control but the effect of fructose on the weights of rats fed with *Ec*N (pAN6) along with fructose was not significant as the rats had body weights similar to the control group (Fig 3.1). Also there was no significant difference in the body weights of rats fed with *Ec*N (pAN6) along with fructose as compared to the Fe Def control.



Groups	1	2	3	4	5	6	7	8	9	10	11
Fructose	-	-	+	+	-	-	+	-	+	-	+
Iron	-	-	-	+	+	+	+	+	+	+	+
Ascorbic Acid	-	-	-	-	+	-	-	-	-	-	-
Probiotic	-	-	-	-	-	EcN-2	EcN-2	EcN (pAN1)	EcN (pAN1)	EcN (pAN6)	EcN (pAN6)

Figure 3.1: Effect of Genetically Engineered Probiotic *E. coli* Nissle 1917 on Body weight. Values are expressed as mean ± SEM (n =5- 6 each group). *P ≤ 0.05 compared with control group.

3.3.2 Serum profile

Confirmation of Iron Deficiency:

One of the primary signs of iron deficiency is characterized by low levels of transferrin bound iron. Here also, Iron deficient group has significantly lower levels of transferrin bound iron in blood as compared to the control group (Fig 3.2). So it can be concluded that iron deficiency and not anemia has been induced.

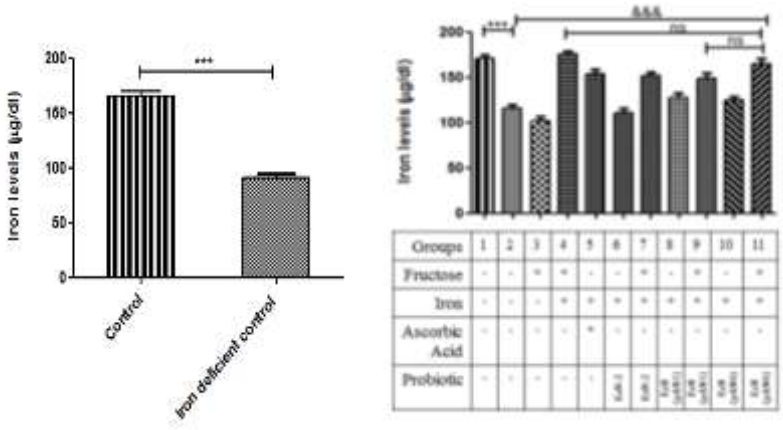


Figure 3.2: Serum Iron levels before and after the repletion period. Values are expressed as mean ± SEM (n=6 each group). ***P≤0.001 compared with control, &&&P≤0.001 compared with iron deficient control.

Tf bound iron levels in Fe Def control group is significantly lower although the rats were given Fe in the diet. As compared to the Fe Def control, the Tf bound Fe levels were restored in the rats which were fed with fructose, ascorbic acid, and in *Ec*N (pAN1) and *Ec*N (pAN6) along with fructose..Fructose also helps in iron absorption but using it alone can't be an option as fructose consumption leads to metabolic syndrome and oxidative stress in the body. So it can be used in combination with *Ec*N (pAN1) or *Ec*N (pAN6). Fructose is known to cause hyperglycemia and dyslipidemia, so fasting blood glucose levels and serum cholesterol and triglyceride levels were estimated.

Fasting Blood Glucose levels were significantly increased in the Fructose control group as compared to the control, whereas, restoration of glucose levels was observed in the groups fed with the probiotics *Ec*N (pAN1) and *Ec*N (pAN6) along with fructose, with *Ec*N (pAN6) being more efficient. Iron deficient control group didn't show any significant changes in the fasting blood glucose levels (Fig 3.3).

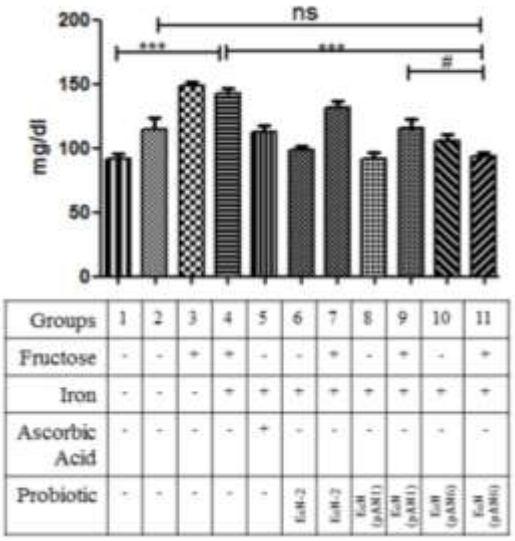


Figure 3.3:Effect of Genetically Engineered Probiotic *E. coli* Nissle 1917 on Fasting Blood Glucose levels.Values are expressed as mean ± SEM (*n* = 5-6 each group). *** P ≤ 0.001 compared with control group, #P ≤ 0.05 compared with (Fructose+iron+*Ec*N(pAN1).

Serum Cholesterol and Triglyceride levels, both were found to be significantly increased in the fructose group as compared to the control group, whereas restoration of both, the levels of cholesterol and triglycerides was seen in the groups fed with *Ec*N(pAN1) and *Ec*N(pAN6) along with fructose, with *Ec*N(pAN6) being more effective (Fig 3.4). There was no significant difference in the cholesterol and triglyceride levels in the Fe Def control group as compared to the normal control group and the group fed with fructose and *Ec*N (pAN6).

3.3.3 Effect of probiotic treatment on Antioxidant status

Since fructose consumption leads to generation of oxidative stress in the liver, and also iron deficiency induces oxidative stress, antioxidant parameters were checked. And it was seen clearly, that catalase and SOD activity were considerably reduced in the fructose group and iron deficient control group, and the levels were almost restored to normal levels in the groups fed with *Ec*N (pAN1) and *Ec*N (pAN6) along with fructose. Also, a significant increase in the lipid peroxidation was seen in the fructose fed group and Fe Def control which was found to be decreased in the *Ec*N (pAN1) and *Ec*N (pAN6) fed groups (Fig 3.5). Reduced Glutathione, which is yet another mechanism for handling oxidative stress in the body, was seen to be decreased in the fructose and Fe Def control

groups, indicating generation of oxidative stress. The Glutathione levels were restored to almost normal in the groups fed with *Ec*N (pAN1) and *Ec*N (pAN6) along with fructose.

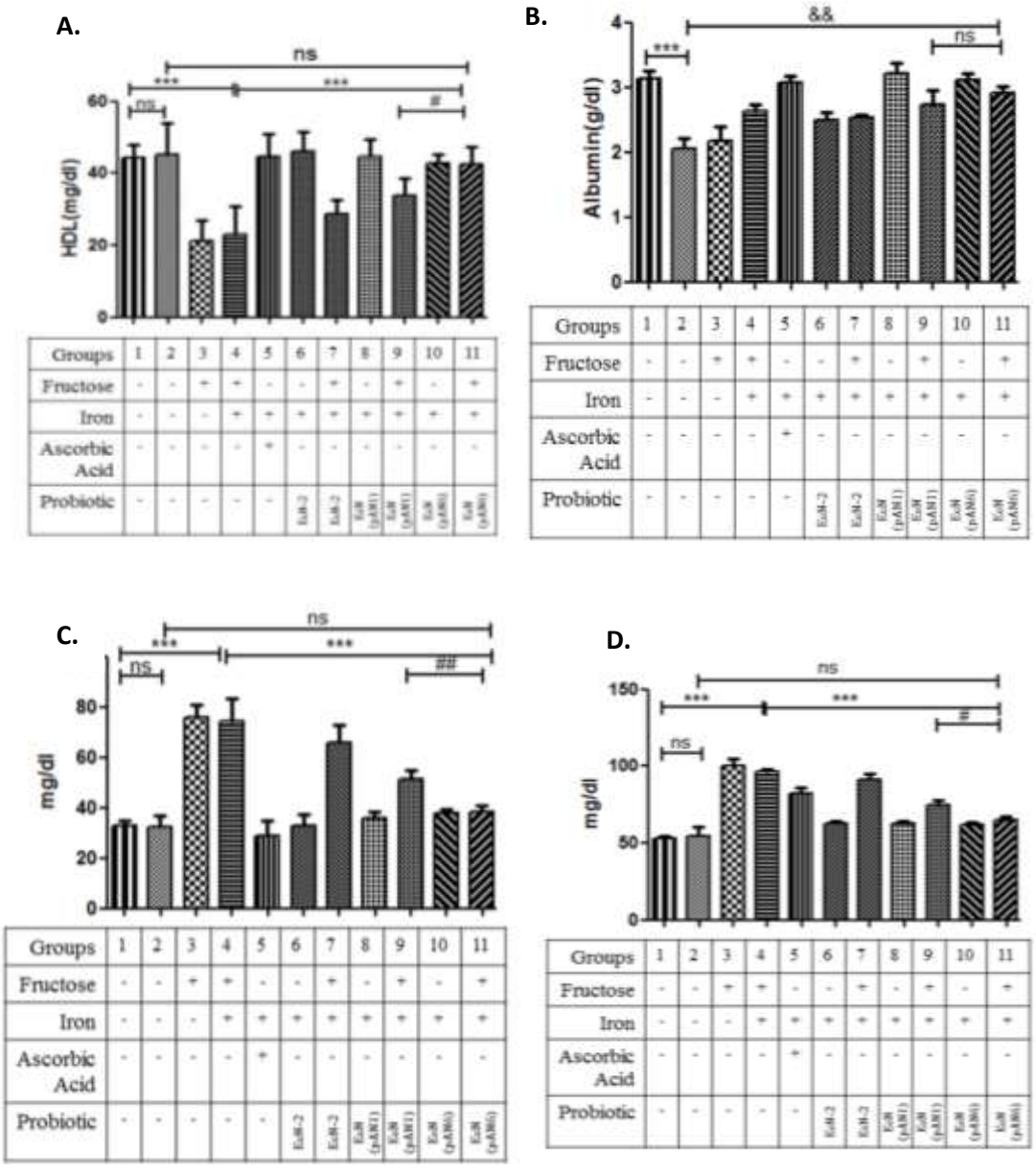


Figure 3.4:Effect of Genetically Engineered Probiotic *E. coli* Nissle 1917 on Serum lipid profile (A)HDL (B)Albumin (C)Cholesterol and (D) Triglyceride levels. Values are expressed as mean ± SEM (n=5-6 each group). ***P≤0.001 compared with fructose control, $^{\#}$P≤0.05, $^{\#\#}$P≤0.01 compared with (Fructose+iron+*Ec*N(pAN1)

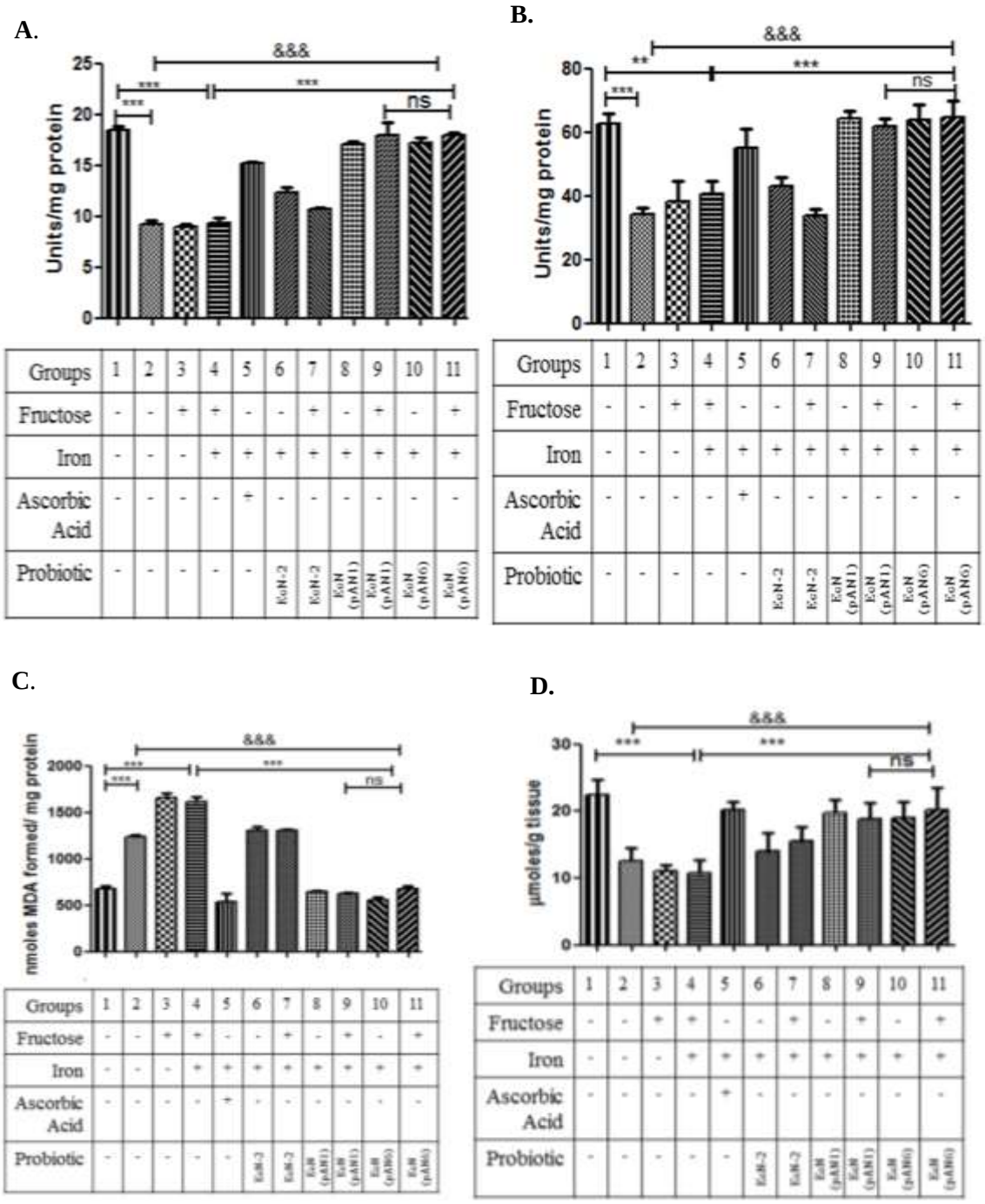


Figure 3. 5: Effect of Genetically Engineered Probiotic *E. coli* Nissle 1917 on Antioxidant status in liver (A) Catalase, (B) SOD activity. (C) Lipid Peroxidation, (D) Reduced Glutathione levels. Values are expressed as mean ± SEM (n=5-6 each group). ***P≤ 0.001, **P≤ 0.01 compared with fructose control; &&&P≤0.001 compared with iron deficient control.

Similar results were obtained for the antioxidant status of blood as were obtained in the antioxidant status of liver. Catalase and SOD activity were significantly reduced and lipid peroxidation was on a higher scale in the fructose and iron deficient control

groups as compared to the normal control group (Fig 3.6). And the levels of catalase and SOD were restored back to almost normal in the probiotic *Ec*N (pAN1) and *Ec*N (pAN6) fed group as compared to the iron deficient control group. And the lipid peroxidation was also reduced in probiotic fed group.

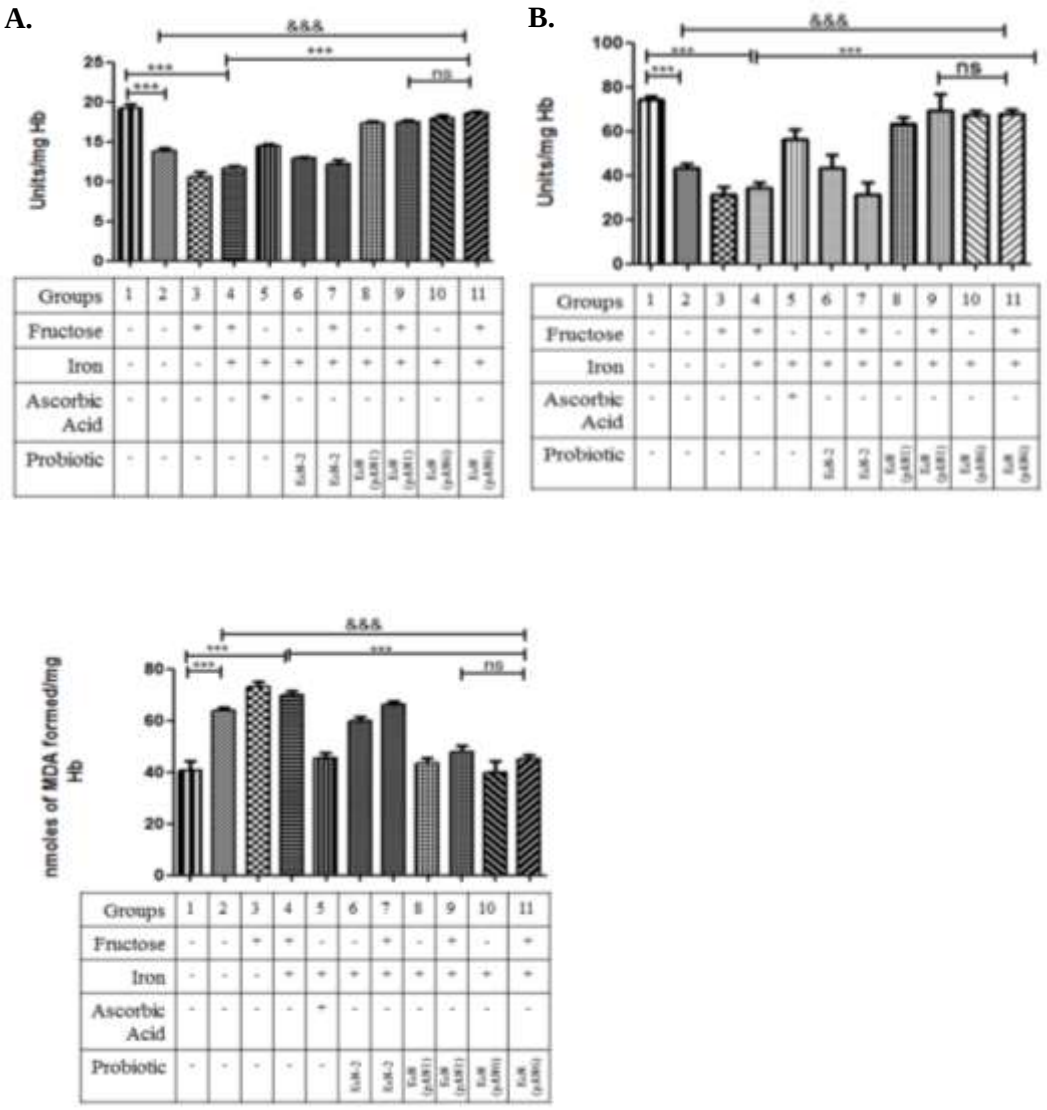


Figure 3.6: Effect of Genetically Engineered Probiotic *E. coli* Nissle 1917 on Antioxidant status of blood (A) Catalase and (B) SOD activity and (C) Lipid Peroxidation. Values are expressed as mean ± SEM (n=5-6 each group). ***P≤ 0.001 compared with fructose control; &&&P≤0.001 compared with iron deficient control.

3.3.4 Effect of probiotic treatment on Liver Function Marker

Since, oxidative stress is induced in the liver due to iron deficiency and fructose consumption, liver function markers i.e. ALT and AST levels in the serum. AST and ALT levels were seen to be increased considerably in the iron deficient and fructose control groups as compared to the normal control and the levels were restored almost back to normal in the probiotic *Ec*N (pAN1) and *Ec*N (pAN6) fed groups, *Ec*N (pAN6) being more efficient (Fig 3.7).

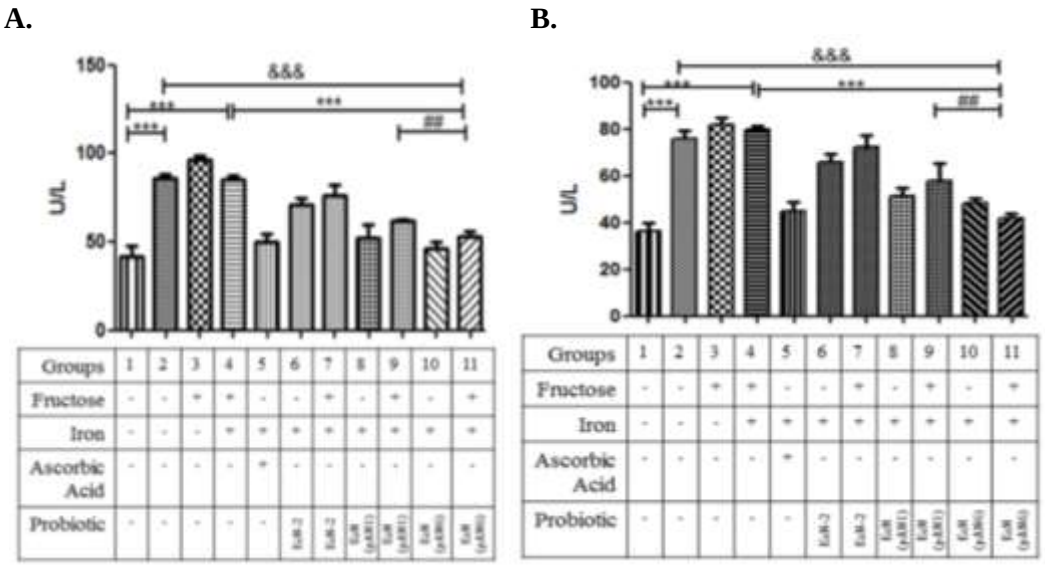


Figure 3.7: Effect of Genetically Engineered Probiotic *E. coli* Nissle 1917 on Liver Function markers (A) ALT and (B) AST in serum. Values are expressed as mean ± SEM (n=5-6 each group). ***P≤ 0.001 compared with fructose control, $^{\#\#}$P ≤ 0.01 compared with Fructose+iron+*Ec*N(pAN1); $^{\&\&\&}$P≤0.001 compared with iron iron deficient control.

3.3.5 Effect of probiotic treatment on mRNA expression

The expression pattern of genes related to iron transport is depicted in Fig.3.8.These genes showed relatively lower expression in iron depleted control group as compared with the nondepleted control.

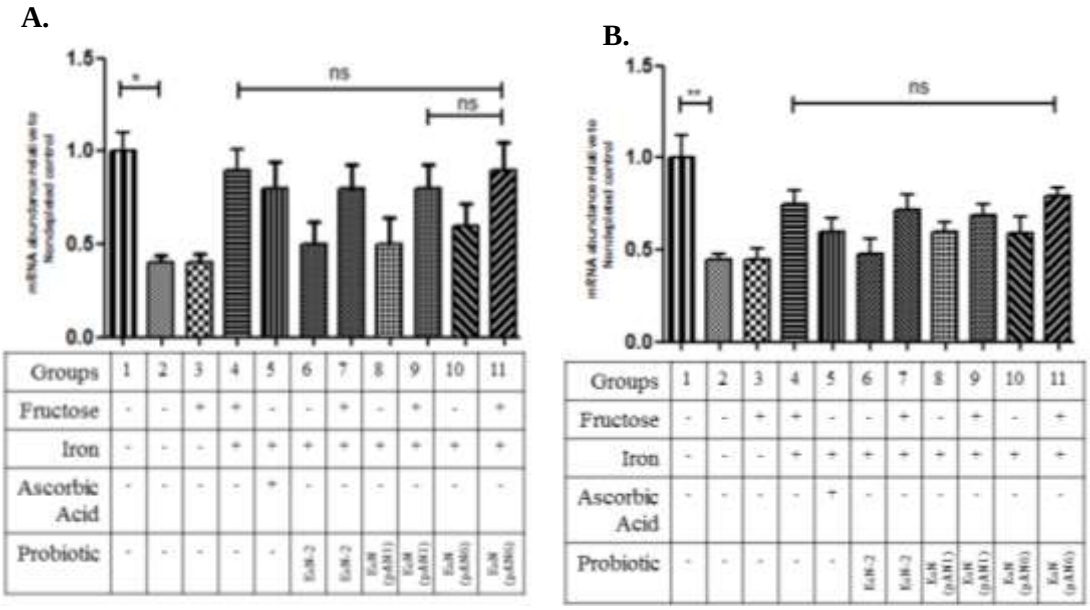


Figure 3.8: Effect of iron depleted diet and repleted diet along with probiotic treatment on expression of iron transporter genes (A) Ferritin (B) Transferrin.

3.3.6 Histopathological analysis.

Histopathological anaylsis showed the accumulation of lipid droplets upon fructose treatment. Supplementation of rats with probiotic *Ec*N (pAN6) showed significant reduction in lipid droplet accumulation (Fig.3.9)**.**

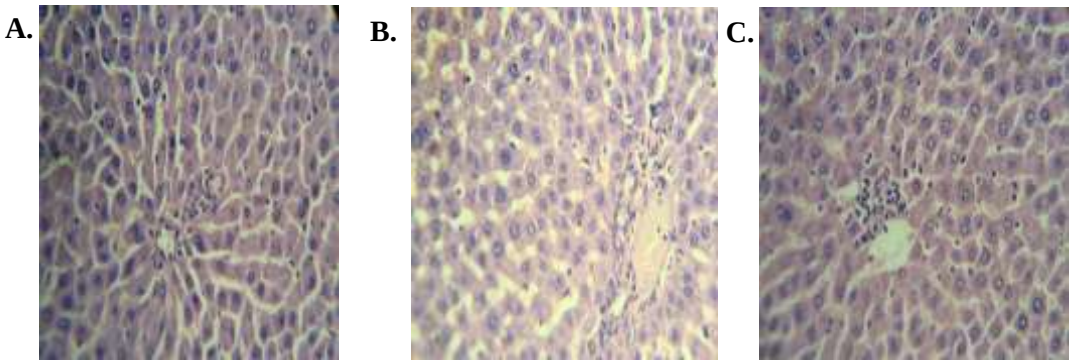


Figure 3. 9: Haematoxylin and Eosin staining of rat liver tissue. Images were taken by LEICA DME microscope at 40 X magnification (A) Control, (B) Fructose(C) *Ec*N (pAN6).

3.4 Discussion

Populations sustaining on plant-based diets have higher iron deficiencies due to inadequate iron intake along with low iron bioavailability that is independent of sex making the strategies to enhance iron absorption by the intake of foods rich in iron, as well as dietary factors important (Hoppe *et al*., 2008). Current strategy for treating iron deficiency is giving oral iron in combination with ascorbic acid, but it has its own limitations (Camaschella, 2015).Rats fed with an AIN-93 Fe Def diet for 10 weeks decreased serum iron content by 57% as compared with nondepleted controls (Saini *et al*., 2014). One of the primary signs of iron deficiency is characterized by low serum iron and haemoglobin concentrations (Reeves, 1997)**.** In the present study, Tf bound Fe levels significantly decreased in the rats fed with the diet after 10 weeks demonstrating establishment of iron deficiency.

Increase in iron absorption by fructose is attributed to the incredible chelating and reducing ability (Dell, 1993). Fructose significantly increases iron-induced ferritin formation in human intestinal cell (Christides *et al*., 2013). But, increase in fructose consumption over time has paralleled with increase in the prevalence of obesity and thus, fructose has also been reported as an obesogen (Lindqvist *et al*., 2008). Iron absorption is enhanced by ascorbic acid (Lynch *et al.,* 2003). When rats were fed with fructose, ascorbic acid, and the *Ec*N-2, *Ec*N (*pqq*), and *Ec*N (*pqq-glf-mtlK*) probiotics after 10 weeks along with iron in the diet for 4 weeks, here also, Tf bound Fe levels increased significantly by administration of fructose, ascorbic acid, *Ec*N (*pqq*) and *Ec*N (*pqq-glf-mtlK*). Although all four treatments can be used for treating iron deficiency, long term oral iron supplementation and fructose consumption could lead to several side effects (Camaschella, 2015).

High fructose intake has deleterious effects on body weight, glucose homeostasis/insulin sensitivity. Fructose can cause hypertriglycidemia and increase intrahepatic lipid levels (Tappy *et al*., 2010)**.** Consumption of High Fructose Corn Syrup-Sweetened beverages increased LDL, TG and cholesterol. Consumption of fructose-sweetened beverages decreased glucose tolerance **(**Schaefer *et al*., 2009; Stanhope *et al.,*

2011). This was also seen in the present study as Glucose, body weight, Cholesterol and TG levels increased in the fructose control group but all the parameters were restored back to almost normal values in the group that was fed with *Ec*N *(pqq-glf-mtlK)* and *Ec*N *(pqq)* and fructose, thus, preventing the dyslipidemia and hyperglycemia that is caused due to fructose intake, with *Ec*N (*pqq-glf-mtlK*) showing more efficiency.

Fe Def rats show higher levels of oxidants and lipid peroxidation (Walter *et al*., 2002) and the iron-deficient patients show MDA levels that are significantly higher than in the controls and the erythrocyte SOD, CAT activities, and GPx levels are significantly lower in iron deficient patients relative to controls (Kurtoglu *et al.*, 2003). Additionally, increase in the catabolism of fructose decreases the level of hepatic antioxidant enzyme activities such as SOD, CAT, and GPx along with reduction of total glutathione levels (Suwannaphet *et al.,* 2010). Present study showed consistently lower SOD and catalase activities in blood and liver and higher levels of lipid peroxidation which was ameliorated in probiotic *Ec*N (*pqq-glf-mtlK*) and *Ec*N (*pqq*) groups.

In accordance with the reported studies, the fructose- fed rat model observed a significant ($p < 0.001$) and marked (93%) increase in AST as compared with control rats (Haeri *et al.,* 2009). Similarly, significantly elevated levels of serum liver enzymes (ALT and AST) were found in the fructose-induced untreated animals (Tanko *et al.,* 2013). Consistent with these reports, significant increase was seen in ALT and AST activities in the serum of iron deficient and fructose control groups indicating liver damage. However, iron deficiency was not seen in the group fed with *Ec*N (*pqq-glf-mtlK*) and *Ec*N (*pqq*), with former being more efficient.

Fructose alone is not an effective strategy for enhancing iron absorption. So, probiotic *Ec*N (*pqq-glf-mtlK*), which can prevent the side effects of fructose consumption, could be better strategy for enhancing iron absorption. Moreover, up taken fructose is converted to mannitol by *Ec*N *(pqq-glf-mtlK),* which is a prebiotic and is fermented by the colonic microflora to produce SCFAs. SCFAs, in turn, are known to facilitate iron absorption by reducing ferric ions into ferrous forms (Freitas *et al*., 2012; Marciano *et al*., 2015). Thus, iron absorption gets enhanced by the beneficial effects of both fructose and SCFA.

3.5 Conclusion

Present study demonstrates that supplementation of probiotic *Ec*N (*pqq-glf-mtlK*) along with fructose could be better strategy than only supplementing fructose for tackling iron deficiency due to combined effect of probiotic, fructose and SCFAs.