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Genetically Engineered *Escherichia coli* Nissle 1917 Synbiotics Reduce Metabolic Effects Induced by Chronic Consumption of Dietary Fructose

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Abbreviations: *Ec*N, *Escherichia coli* Nissle 1917; TG, Triglycerides; Fdh, Fructose dehydrogenase;

Abstract

Aims

To assess protective efficacy of genetically modified *Escherichia coli* Nissle 1917 (*Ec*N) on metabolic effects induced by chronic consumption of dietary fructose.

Materials and Methods

*Ec*N was genetically modified with fructose dehydrogenase (*fdh*) gene for conversion of fructose to 5-keto-D-fructose and mannitol-2-dehydrogenase (*mtl*K) gene for conversion to mannitol, a prebiotic. Charles foster rats weighing 150–200 g were fed with 20% fructose in drinking water for two months. Probiotic treatment of *Ec*N (*pqq*), *Ec*N (*pqq-glf-mtl*K), *Ec*N (*pqq-fdh*) was given once per week 10^9 cells for two months. Furthermore, blood and liver parameters for oxidative stress, dyslipidemia and hyperglycemia were estimated. Fecal samples were collected to determine the production of short chain fatty acids and pyrrolo-quinoline quinone (PQQ) production.

Results

*Ec*N (*pqq-glf-mtl*K), *Ec*N (*pqq-fdh*) transformants were confirmed by restriction digestion and functionality was checked by PQQ estimation and HPLC analysis. There was significant increase in body weight, serum glucose, liver injury markers, lipid profile in serum and liver, and decrease in antioxidant enzyme activity in high-fructose-fed rats. However the rats treated with *Ec*N (*pqq-glf-mtl*K) and *Ec*N (*pqq-fdh*) showed significant reduction in lipid peroxidation along with increase in serum and hepatic antioxidant enzyme activities. Restoration of liver injury marker enzymes was also seen. Increase in short chain fatty acids (SCFA) demonstrated the prebiotic effects of mannitol and gluconic acid.

MTLK, mannitol-2-dehydrogenase; PQQ, pyrroloquinoline quinone; SCFA, short chain fatty acids; GLF, glucose facilitator protein; CFU, colony forming unit; SOD, Superoxide dismutase; PCV, Pack cell volume; CAT, Catalase; GSH, glutathione; AST, Aspartate transaminase; ALP, Alkaline phosphatase; ALT, Alanine transaminase; vgb, *Vitreoscilla* haemoglobin; H_2O_2, Hydrogen peroxide; MDA, Malondialdehyde.

Conclusions

Our study demonstrated the effectiveness of probiotic *Ec*N producing PQQ and fructose metabolizing enzymes against the fructose induced hepatic steatosis suggesting that its potential for use in treating fructose induced metabolic syndrome.

Introduction

Fructose, present in high fructose corn syrup, is known to induce metabolic syndrome leading to type 2 diabetes mellitus, cardiovascular disease and mortality [1, 2]. Fructose is converted to triglycerides (TG) through de novo lipogenesis when taken in excess amount building up lipids in the liver [3]. These lead to elevated blood lipid levels resulting into inflammation, insulin resistance, increased oxidative stress, and blood glucose levels.

High-fructose fed rats develop clinical features of metabolic syndrome and therefore they are used for assessing beneficial effects of various treatments against metabolic syndrome [4]. In rats fed with high fructose diet, probiotic treatment has been shown to significantly reduce oxidative stress, insulin resistance and lipogenesis [5]. Prebiotic such as short chain fatty acids (SCFA) stimulates fatty acid oxidation, inhibits lipogenesis, and glucose production through inhibition of gluconeogenic gene expression [6, 7]. Mannitol, a known prebiotic, converted to SCFA such as butyrate has been demonstrated to confer protection against the development of colon cancer; in the prevention and treatment of the metabolic syndrome [8, 9]. The combination of prebiotic and probiotic known as "synbiotic" synergistically promotes the growth and survival of existing beneficial bacteria along with the newly added probiotic strains in the colon [10].

Pyrroloquinoline quinone (PQQ) is a powerful antioxidant compound synthesized by many Gram negative bacteria but not by humans and human microbiota [11, 12]. It can induce nerve cells regeneration, enhance mitochondrial functions and reproductive capabilities as well as maintain mitochondrial and neuronal function [13]. Our previous work demonstrated that probiotic *E. coli* CFR 16 expressing *Vitreoscilla* haemoglobin (VHb) and secreting PQQ protected against CCl_4 and dimethyl hydrazine induced damage by reducing liver and colon damage mediated by their antioxidant abilities [14, 15] This probiotic treatment also restored neurotransmitter levels which alter in response to oxidative damage [16]. Additionally, probiotic *Ec*N producing PQQ was found to be more effective than orally given PQQ against alcohol and rotenone induced oxidative stress [17, 18].

Uptake of fructose in *E. coli* is mediated by phosphotranferase system (PTS) leading to phosphorylated D-fructose in the cytoplasm [19]. On the other hand, *Zymomonas mobilis* encodes a glucose facilitator protein (GLF) which allows efficient uptake of unphosphorylated D-fructose [20]. We used the genes corresponding to enzymes that catabolize fructose to mannitol and 5-keto-D-fructose in *Ec*N. Heterofermentative Lactic acid bacteria, *Lactobacillus brevis* catalyze the conversion of D-fructose directly to D-mannitol by cytosolic mannitol-2-dehydrogenase (MTLK) [21]. Likewise, *Gluconobacter japonicas* NBRC3260 possess membrane bound Fructose dehydrogenase (FDH; EC 1.1.99.11) which catalyzes the oxidation of D-fructose to 5-keto-D-fructose. FDH is used in the diagnosis and food analysis due to its high substrate specificity to D-fructose [22]. In this study, we genetically modified *Ec*N producing PQQ to convert fructose to 5-KF and mannitol in gut by incorporating fructose metabolizing enzymes, FDH and MTLK along with GLF protein, respectively (S1 and S2 Tables). The potential of these modified probiotics, *Ec*N (*pqq-fdh*) and EcN (*pqq-glf-mtlK*) producing PQQ and

fructose metabolizing enzymes was investigated for ameliorating high fructose induced metabolic syndrome.

Material and Methods

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 S1 and S2 Tables. Routine DNA manipulations were done in *E. coli* DH10B (Invitrogen, USA) using standard molecular biology protocols from Sambrook *et al* [23]. For supplementation to different rat groups, probiotics with different metabolic efficacy were grown overnight in Luria Broth at 37°C, 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.

Construction of pJET with *fdh-pqq* under constitutive *tac* promoter

For the constitutive expression of *fdh* under *tac* promoter, *tac* promoter was obtained by polymerase chain reaction amplification using primers listed in S3 Table. *tac* forward primer contains a modification in *tac* promoter at *lac* repressor binding site and *tac* reverse primer contains a portion of *tac* promoter and complementary region of *fdh*. PCR amplification was done using XT20 polymerase (Thermo Scientific, USA) from plasmid pMALp2. Then, *fdh* gene was amplified using gene specific primers from the genome of *Gluconobacter frauteuri* IFO3260 (S3 Table). The amplicons of size 0.2 kb and 3.7 kb were gel eluted, purified mixed and again amplified using *tac* forward primer and *fdh* reverse primer yielding the final amplicon containing *fdh* under constitutive *tac* promoter (*ctac**-*fdh*) of size 3.9kb. The c*tac**-*fdh* amplicon was gel eluted, purified and ligated in pJET plasmid which gave pAN2. Construct was confirmed by restriction digestion pattern. The activity of the clone was confirmed by fructose dehydrogenase enzyme assay upon transforming in *Ec*N:: *vgb-gfp*. For the constitutive expression of *pqq* operon, *pqq* was amplified by polymerase chain reaction amplification using primers from the genome of *G. suboxydans*. This amplicon c*tac**-*pqq* was gel eluted, purified and ligated in pJET plasmid. Further, pAN2 was linearized using *Xho*I and end-filled. Digestion of pAN1 with *Bgl*II gave *pqq* operon which was gel eluted, purified and ligated in *Xho*I digested and gel purified pAN2 which gave pAN7 construct. The construct was confirmed by restriction digestion pattern and PCR amplification. This was followed by transformation of the final construct pAN7 in *Ec*N:: *vgb-gfp*. The construct was confirmed by restriction digestion pattern and PQQ quantification in the bacterial supernatant using fluorometer (Hitachi High-Technologies Corporation, Japan).

Construction of pJET with *pqq-glf-mtlK* under constitutive *tac* promoter

The strategy for constitutive expression of *mtl*K gene was similar to that of *fdh* gene. The promoter was first amplified by polymerase chain reaction using primers listed in S3 Table. Then, *mtl*K gene was obtained using polymerase chain reaction by primes listed in S3 Table from pRSETmtlK. The amplicon *ctac**-*mtl*K gene was gel eluted, purified and ligated in pJET vector which gave pAN4. The *glf* gene was amplified from *Zymomonas mobilis* using primers listed in S3 Table. The *ctac**-*glf* gene was then gel eluted, purified and ligated in pJET vector which gave pAN3. Digestion of pAN1 by *Bgl*II gave *ctac**-*pqq* gene cluster which was gel eluted, purified and ligated in *Xho*I digested, gel purified pAN3. Construct was confirmed by restriction

digestion pattern. Finally, the *ctac**-*pqq* and *ctac**-*glf* genes were amplified together and the product containing *ctac***pqq*-*ctac***glf* was inserted in *Xho*I digested, gel eluted purified vector pAN4 to give pAN6. This was followed by transformation of the final construct pAN6 in *Ec*N:: *vgb-gfp*. Functionality of construct was confirmed by mannitol-2-dehydrogenase enzyme assay. Briefly the cells grown in M9 minimal medium [23] were harvested aseptically at stationary phase by centrifugation at 9,200 g for 2 min at 4°C. Cell-free extract was prepared by sonication [24]. Mannitol-2-dehydrogenase activity was assayed by measuring the rate of oxidation of NAD(P)H using fructose as substrate according to Liu *et al.* [21]. The culture supernatant collected at the end was used for gluconic acid estimation produced by *Ec*N (pAN7) and *Ec*N (pAN6) by HPLC [24].

PQQ quantification

PQQ was extracted from E.*coli* strains and liver tissue as described in Suzuki *et al.* [25] and Singh *et al.* [17]. Briefly the cells were grown overnight in M9 minimal medium containing glucose. These cells were harvested, and the supernatant was used for PQQ extraction. Culture supernatant was incubated with 50% acetonitrile at 65°C for 2 hours followed by centrifugation at 15,000xg for 10 minutes. The clear supernatant attained was dried using concentrator in vacuum. The residues were dissolved in 50% n-butanol (1 mg/ml) and incubated at 50°C till it dries. Finally, the residues attained were dissolved in water and filtered with 0.2 micron filter. Quantification was done fluorimetrically as described by Suzuki and colleagues (1990) using Hitachi fluorescence spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) with excitation 375 nm and emission 465 nm (uncorrected). Standard plot for area under curve was drawn using 6 different concentration of standard PQQ ranging from 0.2 to 20 μM. Liver tissue and colonic contents were homogenized in phosphate-buffered saline 20 and 10% (w/v), respectively followed by centrifugation at 10,000 xg for 20 minutes to obtain supernatant for PQQ extraction followed by quantification as described above.

Animals

Adult albino male Charles Foster rats (180–200 g) were used for animal studies. All rats were housed in plastic cages and maintained in controlled condition as per committee guidelines *i.e.* temperature (25 ± 1°C), 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. The presented research was approved by Animal Ethical Committee of Department of Biochemistry, The M. S. University of Baroda, Gujarat, India (Approval No. 938/a/06/CPCSEA), and CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals). The rats were acclimated to laboratory conditions, monitored daily twice for welfare without disturbing and checked whether each rat is feeding and drinking. Rats were checked for any red staining around the eyes and physically examined by running fingers over their body to check whether they are normal. Rat body weight and food consumption were measured weekly.

Experimental design

For present study, rats were divided into seven different groups ($n = 6$) as follows: Control group received pellet diet; Fructose group received pellet diet and 20% fructose in drinking water; F + *Ec*N-2 group received pellet diet, 20% fructose in drinking water and *Ec*N-2; F + *Ec*N (*pqq*) group received pellet diet, 20% fructose in drinking water and *Ec*N (*pqq*); F + *Ec*N (*pqq-glf*) group received pellet diet, 20% fructose in drinking water and *Ec*N (*pqq-glf*); F + *Ec*N (*pqq-*

glf-mtlK) group received pellet diet, 20% fructose in drinking water and *Ec*N (*pqq-glf-mtl*K); F + *Ec*N (*pqq-fdh*) group received pellet diet, 20% fructose in drinking water and *Ec*N (*pqq-fdh*). All groups except Control group and Fructose Control group were gavaged with probiotics (10^9 *cfu*) once per week till two months as per Singh *et al.* [17].

Colonic SCFA extraction and quantification

Colonic SCFA extraction and quantification was carried out as described in Singh *et al.* [17]. In brief, after dissection of rats colonic content was collected immediately and stored at -80°C till use. For quantification, samples were taken from -80°C and suspended in sterile deionized water containing 0.015 M H_2SO_4 and detected using Shimadzu HPLC system with C-18 column. (Shimadzu Analytical (India) Pvt.Ltd, Mumbai, India.

Preparation of cell lysate and tissue homogenates

Blood was collected by orbital sinus puncture under general anaesthesia with ether in EDTA coated and normal tubes followed by centrifugation at 1500 g for 10 minutes. Plasma and serum were collected separately in fresh tubes and stored in -80°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 by cervical dislocation Liver homogenates were prepared in different buffers for antioxidant enzyme activity.

Biochemical assays

Superoxide dismutase (SOD) activity was measured by a method which is based on auto-oxidation of pyrogallol monitored spectrophotometrically at 420 nm [26]. Catalase (CAT) activity were monitored by measuring rate of disappearance of hydrogen peroxide (H_2O_2) spectrophotometrically at 240 nm [27]. SOD and CAT activities were reported as units/mg protein. Reduced glutathione (GSH) was performed as described in Beutler *et al.* [28]. Lipid peroxidation was estimated by measuring levels of malondialdehyde at 412 nm as described in Buege *et al.* [29].

Liver enzyme test, kidney function test, and lipid estimation

Aspartate transaminase(AST), Alkaline phosphatase(ALP), and Alanine transaminase(ALT), Total bilirubin, triglyceride, HDL, VLDL and total cholesterol content in blood plasma were measured using kits as per manufacturer protocol (Beacon Diagnostics Pvt. Ltd. Navsari, India).

mRNA expression and qRT-PCR

RNA was extracted with Trizol (Invitrogen Bio Services India Pvt. Ltd., Bangalore, India) and cDNAs were generated from 1 μg total RNA (Reverse Transcription Kit; Applied Bio systems, Foster City, CA) following the manufacturer's instructions. The primers for fatty acid synthase and acyl coenzyme A gene were ACCTCATCACTAGAAGCCACC AG (forward) and GTGG *TAC*TTGGCCTTGGGTTTA (reverse), and CCCAAGACCCAAGAGTTCATTC (forward) and TCACGGATAGG GACAACAAAGG (reverse), respectively. PCR was performed using ABI Quant-StudioTM 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 thermo cycler (QuantStudioTM). Each

sample was analysed in duplicate. The mean cycle time of the linear part of the curve was designated Ct.

Histopathological changes

Liver tissue was fixed in 10% neutral buffered formalin. Histological sections were stained with hematoxylin and eosin and evaluated by pathologist unaware with experimental codes.

Statistical analysis

All values are expressed as mean ± 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 performed using commercially available statistical software packages (Graph Pad PRISM Version 5.0 La Jolla, CA 92037 USA).

Results

Cloning and expression of *pqq-glf-mtl*K and *pqq-fdh* in *Ec*N::*vgb-gfp*

The pAN1 plasmid possessing *G. suboxydans pqq* operon, the pAN2 plasmid containing *G. frauteuri* IFO3260 *fdh* gene cluster, the pAN3 plasmid harbouring *Z. mobilis glf* gene, the pAN4 plasmid containing *L. brevis mtlK* gene, the pAN5 plasmid possessing *pqq* operon and *glf* gene together, the pAN6 plasmid containing *pqq* operon, *glf* gene and *mtlK* together, the pAN7 plasmid harbouring *pqq* operon and *fdh* together under constitutive *tac* promoter were cloned in pJET vector (S1 Fig). Functional confirmation of *Ec*N transformants harbouring pAN1, pAN5, and pAN6 and pAN7 plasmids was done by growth and acidification on Tris-buffered medium containing methyl red as pH indicator [30].

In M9 medium, *Ec*N containing pAN1, pAN5, pAN6 and pAN7 produced 6.5 ± 0.15 μg / ml, 6.29 ± 0.22 μg / ml, 5.78 ± 0.18 μg / ml and 5.23 ± 0.32μg / ml of PQQ/ml along with 34.44 ± 0.09 mM, 31.44± 0.17 mM, 30.56± 0.24 mM and 31.34 ± 0.19 mM of gluconic acid in the culture medium. Acidification of Tris-buffered medium with methyl red as indicator is due to secretion of gluconic acid by PQQ dependent glucose dehydrogenase (Data not provided). In M9 medium, *Ec*N harbouring *mtlK* and *pqq-glf-mtlK* showed 0.23± 0.22 and 0.2± 0.16 μmoles of NADPH oxidized per min per mg of protein enzyme activity.

Effect of probiotic treatment on body weight, hyperglycemia

The body weight gain of fructose fed group significantly increased compared to control group. After administration of probiotic producing fructose metabolizing enzymes, *Ec*N (*pqq-glf-mtlK*) and *Ec*N (*pqq-fdh*) body weight gain significantly decreased and reached the levels similar to the control group (Table 1). The fasting serum concentrations of glucose were also significantly higher in fructose fed group compared to control group. However, the increase in serum concentrations of glucose, in fructose fed rats were significantly decreased after administration of probiotic producing fructose metabolizing enzymes (Table 1).

Effect of probiotic treatment on hepatic injury markers

Increased serum levels of Aspartate transaminase (AST), Alkaline phosphatase (ALP), and Alanine transaminase (ALT) were found in fructose fed group (Fig 1A–1C). These results showed that fructose induced damage to liver cells. However, supplementation of probiotic producing fructose metabolizing enzymes significantly decreased ALP, AST and ALT levels in fructose fed group.

Table 1. Effect of different probiotic treatment on body weight gain, food intake, fasting glucose, serum insulin levels of rats.

Groups	Body weight gain (g)	Food intake (g/day)	Fasting glucose (mg/dl)	Serum insulin (μg/l)
Control (No F)	43.33±8.45	18.15±1.19	98.04±3.59	1.10±0.21
Fructose control (F)	92.6±8.902**	17.24±1.73	188.48±6.83***	3.14 ± 0.25**
F+ *Ec*N-2	87±9.08**	17.66±1.14	185.57±4.81***	3.28± 0.35**
F+ *Ec*N (*pqq*)	67.75±9.12**	17.77±1.25	154.33±5.34***	2.91± 0.15**
F+ *Ec*N (*pqq-glf*)	66.75±9.23**	17.69±1.58	149.82±10.31***	2.88 ± 0.21**
F+ *Ec*N (*pqq-glf-mtl*K)	52.4±7.31##	17.33±1.93	105.23±10.49**##	1.23± 0.35##
F+ *Ec*N (*pqq-fdh*)	60.75±8.93##	17.97±1.25	120.29±6.50**##	1.57± 0.35##

F, Fructose.

Values are expressed as mean ± SEM ($n = 6$ each group).

** $P \leq 0.01$ and

*** $P \leq 0.001$ compared with control group.

$P \leq 0.01$ compared with fructose control group.

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Effect of probiotic treatment on kidney function

There is a noticeable increase in urea and creatinine in kidneys of rats fed with fructose fed group compared to control group. Treatment with probiotic producing fructose metabolizing enzymes, *Ec*N(*pqq-glf-mtlK*) and *Ec*N(*pqq-fdh*) decreased urea and creatinine in probiotic treated rats as compared to fructose fed group (Fig 1D and 1E).

Effect of probiotic treatment on dyslipidemia

Higher serum levels of LDL, VLDL, cholesterol and TG were noticed in fructose fed group as compared to control group (Fig 2A, 2C, 2D and 2E). Administration of probiotic producing fructose metabolizing enzymes, significantly reduced the increased levels of LDL, TG, VLDL and cholesterol as compared to fructose fed group. Our results demonstrated that higher levels of hepatic TG and cholesterol were found in fructose fed group as compared to control group. Feeding of probiotic producing fructose metabolizing enzymes significantly suppressed the increased hepatic TG and cholesterol levels compared to fructose fed group which showed that probiotic can suppress the formation of hepatic steatosis (Fig 2F and 2G).

Effect of probiotic treatment on Hepatic Fatty Acid Synthase (FAS) and Acyl Coenzyme A Oxidase (ACOx) mRNA Expression

Finally, mRNA expression of FAS and ACOx showed increased and decreased expression, respectively, in fructose fed group (Fig 2H and 2I). This expression was reversed in groups supplemented with probiotic producing fructose metabolizing enzymes.

Effect of probiotic treatment on oxidative stress

Fructose control group rats showed markedly high levels of lipid peroxidation as compared to control rats (Fig 3A) After administration of probiotic producing fructose metabolizing enzymes, the levels of MDA were significantly lowered to near normal levels as compared to untreated fructose fed rats group. Catalase and SOD activities in rat livers were decreased in fructose fed group, as compared to control group (Fig 3B and 3C). Probiotic treatment significantly up regulated these two antioxidant enzyme activities, which were decreased in fructose fed group, in rat liver. Glutathione levels decreased in fructose fed rats compared to control

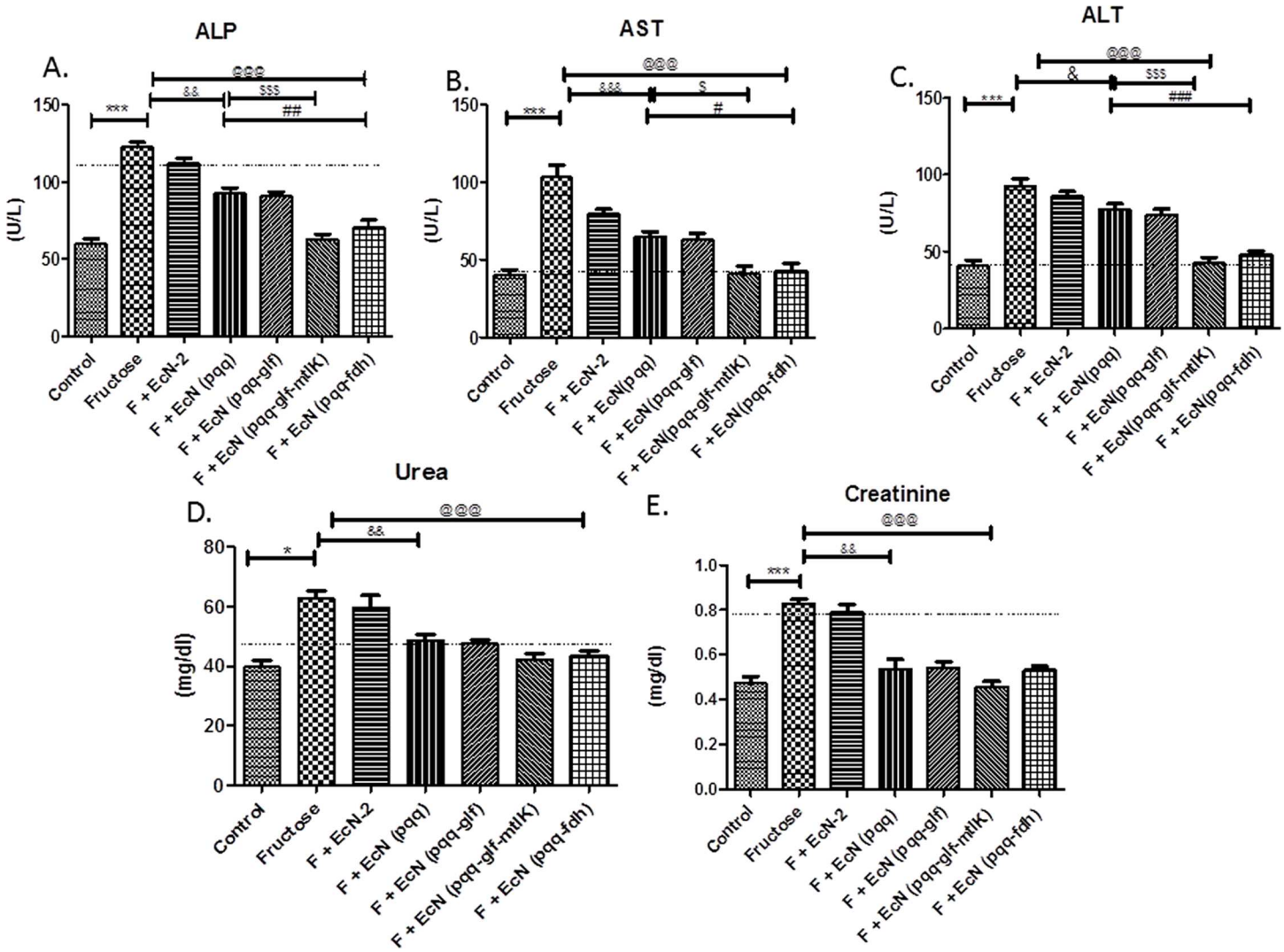

Fig 1. Effect of probiotic treatment on liver enzyme tests. (A) ALP (B) AST (C) ALT activity and kidney function tests (D) Urea (E) Creatinine of serum. Values are expressed as mean ± SEM (n = 5–6 each group). ***P≤ 0.001 compared with fructose control, @@@P ≤ 0.001 compared to fructose control, $$$ P ≤ 0.001, $$ P ≤ 0.01 compared to EcN (*pqq*) group. F: Fructose.

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group (Table 2). Treatment with probiotic producing fructose metabolizing enzymes increased glutathione levels as compared to fructose fed rats.

Effect of probiotic treatment on colonic SCFAs concentration

Additionally, levels of colonic SCFA *i.e.* acetate, propionate and butyrate were found to be increased in EcN (*pqq*), EcN (*pqq-glf*), EcN (*pqq-glf-mtlK*) and EcN (*pqq-fdh*) fed rats in comparison with control rats and fructose fed rats (Table 2).

Effect of probiotic treatment on PQQ concentration in faecal matter and liver

PQQ concentration estimated in faecal matter and in liver of *Ec*N (*pqq*), *Ec*N (*pqq-glf*), *Ec*N (*pqq-glf-mtlK*) and *Ec*N (*pqq-fdh*) co-treated rats, on the day of sacrifice, was found to be

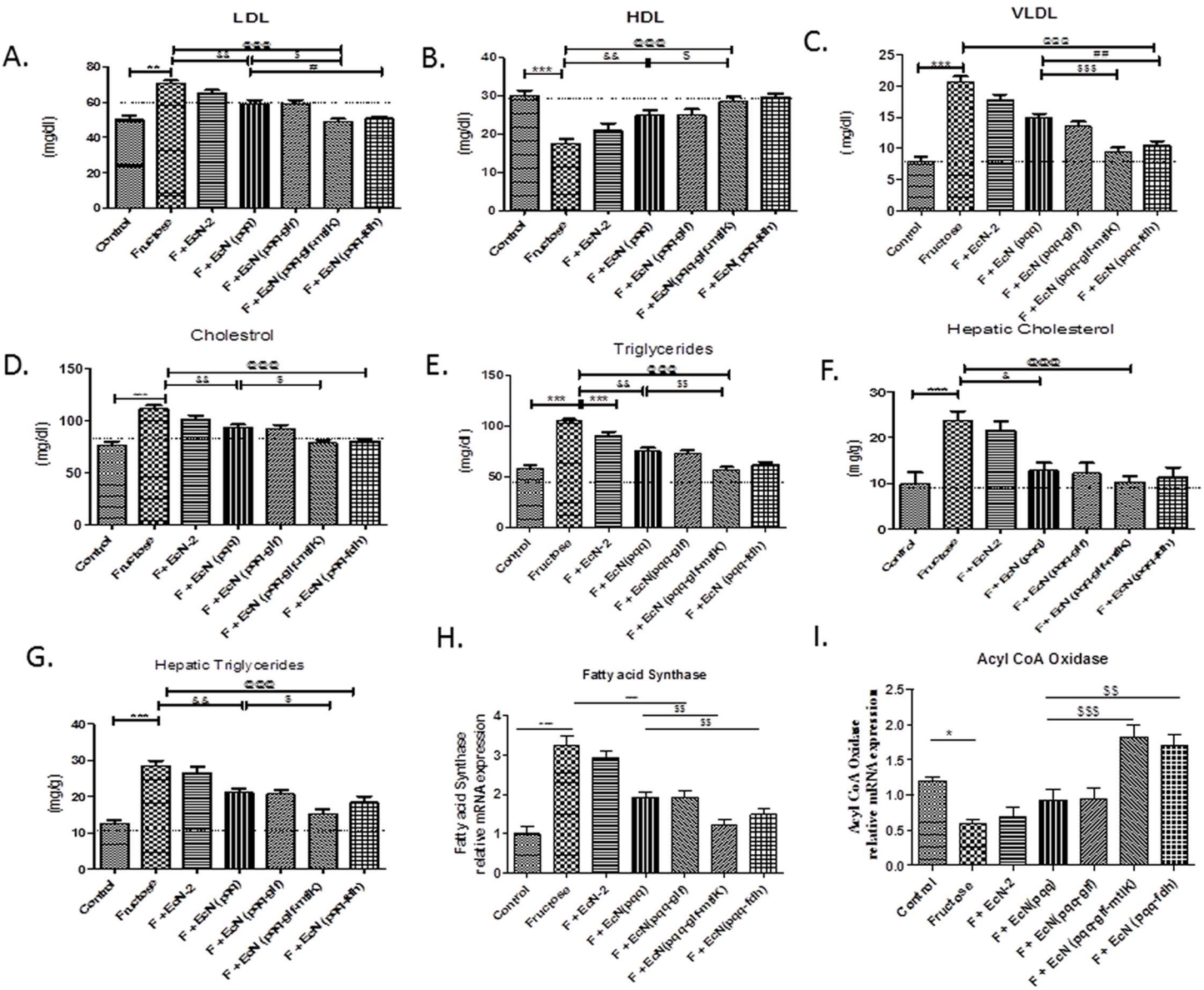

Fig 2. Effect of probiotic treatment on serum lipid profile. (A) LDL, (B) HDL, (C) VLDL, (D) Cholesterol and (E) Triglycerides; on hepatic lipid profile (F) Cholesterol and (G) Triglycerides; (H) mRNA of Fatty acid synthase and (I) mRNA of Acyl Coenzyme A oxidase. Values are expressed as mean ± SEM (n = 5–6 each group). ***P≤ 0.001 compared with fructose control, $^{@@@}$P ≤ 0.001 compared to fructose control, $^{\$\$\$}$ P ≤ 0.001, $^{\$\$}$ P ≤ 0.01 compared to *Ec*N (*pqq*) group, $^{\#\#\#}$ ≤ 0.001. Values are expressed in mg/dl. F: Fructose.

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increased than control and fructose-treated rats (Table 2). Rats treated with *Ec*N-2 and fructose fed rats had similar PQQ concentration.

Histopathological analysis

High degree of lipid droplet accumulation was found in Group 2 rats while Group 6 and 7 rats, supplemented with probiotic producing fructose metabolizing enzymes, showed significant reduction in lipid droplet accumulation which was correlated with decreased serum and hepatic triglyceride levels (S2 Fig).

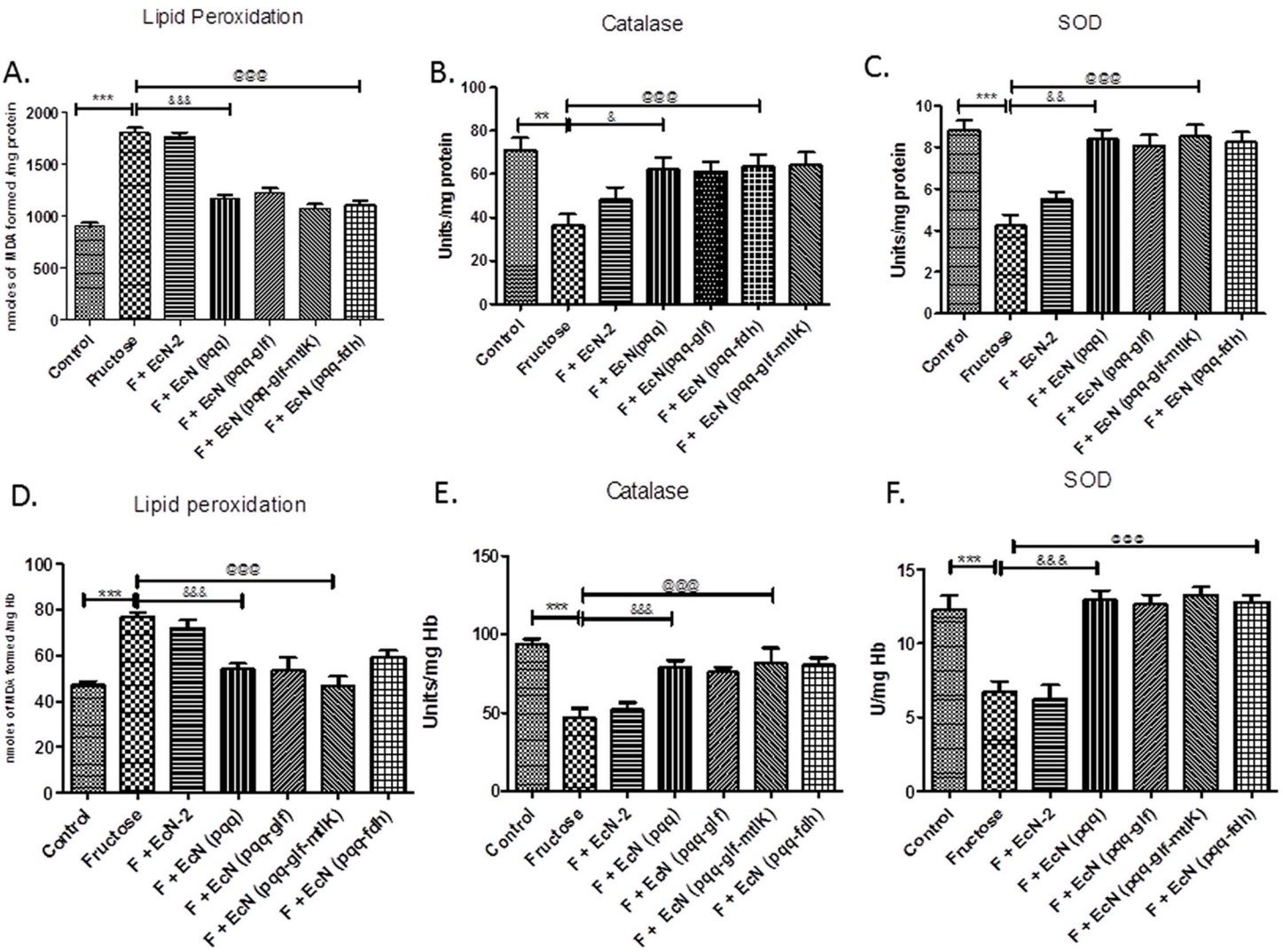

Fig 3. Effect of probiotic treatment on antioxidant status of liver (A), (B) and (C) and blood (D), (E) and (F) in rats. (A and D) Lipid peroxidation (LPO), (B and E) Catalase and (C and F) SOD. Values are expressed as mean ± SEM (n = 5–6 each group). ***P≤ 0.001 compared with fructose control, @@@P ≤ 0.001 compared to fructose control, $ $ $ P ≤ 0.001, $$ P ≤ 0.01 compared to *Ec*N (*pqq*) group. F: Fructose.

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Discussion

Fructose which is highly lipogenic has now become a major constituent of our modern diet even though it was absent in our diet few hundred years ago [31]. In several studies it has been observed that acute fructose ingestion contributes to the synthesis of hepatic triose-phosphate leading to fatty acid synthesis [32].

S3 and S4 Figs. show the proposed mechanism of conversion of fructose by probiotic producing fructose metabolizing enzymes, *Ec*N (*pqq-glf-mtlK*) and *Ec*N (*pqq-fdh*). Fructose is taken up by probiotic *Ec*N (*pqq-glf-mtlK*) through GLF in unphosphorylated form whereby the cytosolic MTLK then converts it to mannitol which is exported outside the *Ec*N (*pqq-glf-mtl*K). The membrane bound fructose dehydrogenase converts the fructose to 5-KF which is then exported outside the EcN (*pqq-fdh*).

Fructose is well known for inducing metabolic syndrome. 20% fructose increased fasting glucose similar to the report of Mamikutty *et al.* [33]. The efficiency of probiotics in

Table 2. Effect of probiotic treatment on Colonic SCFA, PQQ concentration in liver tissue and fecal samples and liver GSH levels in rats.

Groups	SCFA levels			PQQ levels		Liver GSH(μmoles/g tissue)
	Acetate	Propionate	Butyrate	Feacal (n moles /g faecal wet weight)	Liver (picomoles / g tissue)	
Control (No F)	98.43±3.28	18.52±4.24	9.52±1.41	0.698±0.10	30.93±1.13	22.41±1.15
Fructose control (F)	80.32±6.94*	20.54±3.67	11.10±1.41	0.656±0.09	34.23±2.34	11.66±1.24**
F+ *Ec*N-2	76.45 ±4.89	25.53 ± 5.11	10.99±2.91	0.701±0.11	39.23±1.54	14.9 ±3.1**
F+ *Ec*N (*pqq*)	123.12 ±7.61##	28.16±3.65#	18.54 ±2.56#	4.181±0.13**	58.24±4.08**	17.54 ±2.06**
F+ *Ec*N (*pqq-glf*)	120.32 ±4.5##	29.95618#	19.24 ±4.34#	3.633±0.04**	57.89±.94**	16.94±1.75**
F+ *Ec*N (*pqq-glf-mtl*K)	125.27 ±3.45##	30.57512#	20.56±3.4#	3.51± 0.09**	60.32±1.97**	19.23±1.86##
F+ *Ec*N (*pqq-fdh*)	126.12 ±6.61##	28.96±3.65#	19.84±3.9#	3.69± 0.09**	52.43±0.97**	18.93±1.66##

F, Fructose; SCFA, Short chain fatty acid; GSH, Reduced glutathione; PQQ, pyrroloquinoline quinone.

Values are expressed as mean ± SEM ($n = 6$ each group).

*P $\leq$ 0.05 and

** P $\leq$ 0.01 compared with control group.

P $\leq$ 0.05 and

##P $\leq$ 0.001 compared with Fructose control group.

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ameliorating metabolic disorders has been revealed in a high-fructose-fed rat model [14]. Probiotic producing fructose metabolizing enzymes treatment maintained body weight and fasting glucose level in comparison with fructose control group. In our present data, the serum levels of two critical markers of liver injury, ALT and AST, were increased in fructose control rats. The activities of hepatic antioxidant enzymes, SOD and catalase were decreased in fructose control rats. These results demonstrated that high fructose led to production of enhanced oxidative stress in rats, which then resulted in liver damage in fructose fed rats seen in our study. After treatment with probiotic, up regulation of hepatic activities of antioxidant enzymes and down regulation of serum AST and ALT levels in high fructose rats were noticed. These findings implied that consumption of probiotic significantly reduced both liver damage and oxidative stress in fructose fed rats by enhancing hepatic antioxidants expressions and uptake and conversion of fructose in the intestine by probiotic.

High fructose increased plasma TGs, most probably by up regulation of hepatic de novo lipogenesis and secretion of TGs [34]. Administration of probiotic producing fructose metabolizing enzymes significantly reduced the levels of important components of metabolic disorder, including LDL, TG, and cholesterol, enhanced by fructose. In addition to serum levels, the increased hepatic lipid accumulation by fructose was also found to be suppressed by oral administration of probiotic producing fructose metabolizing enzymes. The levels of SCFA increased in probiotic treated group suggest that fructose is converted to mannitol which is further converted to SCFA in intestine by colonic flora. This is supported by the fact that mannitol treatment increased levels of butyrate in large intestine of pigs [12]. Presence of PQQ enables glucose dehydrogenase to produce gluconic acid which in turn is utilized by *Bifidobacteria* and *Lactobacilli* species leading to the formation of SCFA [35].

*Ec*N is known to have many probiotic properties, acts as a safe carrier for localized delivery of biomolecules in intestine for human use [36]. S5 Fig shows the proposed mechanism of action of probiotic producing PQQ and fructose metabolizing enzyme, *Ec*N (*pqq-glf-mtlK*).

*Ec*N possessing MTLK converts fructose to mannitol while secreted PQQ acts as an antioxidant molecule as well as a co-factor for glucose dehydrogenase enzyme which catalyzes the production of gluconic acid. Thus, *Ec*N (*pqq-glf-mtlK*) facilitates the formation of two prebiotic molecules, mannitol and gluconic acid. Mannitol and gluconic acid are metabolized by lactic acid bacteria in lower part of gastrointestinal tract resulting in production of SCFA [12, 35]. Vgb produced by *Ec*N could improve the survival in intestine as seen in probiotic *E. coli* CFR16 strain [14]. Overall metabolic effects observed in the present study demonstrates the synergistic effects of *Ec*N, PQQ, Vgb and SCFA formed due to in situ generation of prebiotic gluconic acid and mannitol. Thus, these *Ec*N probiotics act as synbiotics in the intestine.

Recent studies have recommended that the incorporation of a synbiotic with antioxidants can help in alleviating certain disease states *via* synergistically improved intestinal microflora [37, 38, 39]. Use of genetically modified probiotics is more beneficial than wild-type probiotics by not only acting as a suitable vehicle for the delivery of small molecules but also colonize more efficiently in the gut as most probiotic bacteria do not colonize properly in unhealthy individuals [40]. This is supported by the fact that *Ec*N secreting PQQ was very efficient in preventing adverse effects of chronic ethanol consumption [18]. Thus, this approach may be very useful in developing novel synbiotics for treatment of chronic medical conditions including obesity.

Our study supports the concept of sustained delivery of molecules for treatment of fructose induced hepatic steatosis. This will decrease the need for daily administration of molecules whereby it can be effective strategy in the treatment of metabolic syndrome.

Supporting Information

S1 Fig. Agarose gel analysis of PCR amplicons of the recombinant plasmids (A) pAN1, (B) pAN5, (C) pAN6 and (D) pAN7.
(TIF)

S2 Fig. Haematoxylin and Eosin staining of rat liver tissue. Blue arrows indicate the accumulation of lipid droplets in Hepatocytes of fructose fed rat (Fructose control). Images were taken by LEICA DME microscope at 40 X magnification (A) Control, (B) Fructose control, (C) EcN-2, (D) EcN (*pqq-glf-mtlK*) and (E) EcN (*pqq-fdh*).
(TIF)

S3 Fig. Proposed mechanism of fructose conversion to mannitol by probiotic *Ec*N (*pqq-glf-mtlK*). PTS: Phosphotransferase system, MtlK: Mannitol dehydrogenase.
(TIF)

S4 Fig. Proposed mechanism of fructose conversion to 5-KF by probiotic *Ec*N (*pqq-fdh*). 5-KF: 5-Ketofructose, FDH: Fructose dehydrogenase.
(TIF)

S5 Fig. Proposed mechanism of probiotic *Ec*N (*pqq-glf-mtlK*) producing PQQ and mannitol dehydrogenase in gut on fructose induced metabolic effects.
(TIF)

S1 Table. Plasmids used in this study. Ap = Ampicillin, r = resistance.
(DOCX)

S2 Table. Bacterial strains used in this study.
(DOCX)

S3 Table. List of primers used in this study. [a]Primers used for construction of *ptac**-*pqq-fdh*.[b]Primers used for construction of *ptac**-*pqq—glf-mtlK*.
(DOCX)

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Basic nutritional investigation

Amelioration of cadmium- and mercury-induced liver and kidney damage in rats by genetically engineered probiotic *Escherichia coli* Nissle 1917 producing pyrroloquinoline quinone with oral supplementation of citric acid

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ABSTRACT

Objective: Antioxidants, chelating agents, and probiotics are used to manage the toxic effects of cadmium (Cd) and mercury (Hg). The aim of this study was to investigate the combined effects of antioxidants, chelating agents, and probiotics against heavy metal toxicity.

Method: Genetically modified probiotic *Escherichia coli* Nissle 1917 (*Ec*N-20) producing a potent water soluble antioxidant pyrroloquinoline quinone (PQQ) was supplemented with oral citric acid and compared with another genetically modified probiotic *Ec*N-21 producing PQQ and citric acid against oxidative stress induced by Cd and Hg. Rats were independently given 100 ppm Cd and 80 ppm Hg in drinking water for 4 wk.

Results: *Ec*N-20 was found to be more effective than *Ec*N-2 (*Ec*N strain with genomic integration of *vgb* and *gfp* genes) with orally given PQQ against oxidative stress induced by Cd and Hg. *Ec*N-20 supplemented with oral citric acid was more effective against Cd and Hg toxicity compared with *Ec*N-2+citric acid (oral), *Ec*N-2+PQQ (oral), *Ec*N-2+PQQ (oral)+citric acid (oral), *Ec*N-20, and *Ec*N-21. However, protection shown by *Ec*N-21 was similar to *Ec*N-20.

Conclusion: The combination therapy involving probiotic *Ec*N-20 producing PQQ with citric acid given orally was found to be a moderately effective strategy against toxicity induced by Cd and Hg, whereas the protective effect of *Ec*N-21 was the same as *Ec*N-20.

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Introduction

Liver and kidney are immensely sensitive to the injurious effect of environmental heavy metal pollutants like cadmium (Cd) and mercury (Hg) [1]. Heavy metal pathogenesis is mainly mediated by oxidative stress. Cd and Hg are known to have higher electron affinities for the thiol group, causing inactivation of thiol-containing antioxidants and enzymes by binding them [2]. Currently available physical and chemical methods, as well as bioremediation processes for heavy metal detoxification, are either ineffective or too expensive [3]. The disturbed ratio of pro-oxidant to antioxidant due to oxidative stress can be rebalanced by using antioxidants like vitamin C, carotenoids, selenium, and vitamin E [4]. Pyrroloquinoline quinone (PQQ) is a potent antioxidant compound with redox catalytic cycles of 20 000 [5]. PQQ is more efficient than α-tocopherol and vitamin C, acts as growth factor, enhances reproductive capabilities, and maintains mitochondrial and neuronal function [5–7]. It also reduces inflammation and liver fibrosis [8,9].

The strategy is to overcome the metal toxicity via chelation therapy, where chelators bind with metals to form complexes to enhance their excretion out from body [10]. Sodium citrate was found to be effective in reducing the lead levels to normal or near normal in lead-poisioned humans [11]. Combination therapy involving supplementation of antioxidants with chelation therapy was found to be more effective in reestablishing altered antioxidant status induced by heavy metal toxicity [12–16]. Animal experiments have suggested that physiological recoveries,

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Table 1
Primers

Genes		
tac promoter	F1 (forward)	CC CTCGAG GG TTGACAATTAATCATCGGCTCGTATAATGGATCG AAT TGT GAG
	R1 (reverse) (overlapping region of *pqq* gene cluster)	CCA GGCCAT AAT CTA TGG TCC TTG TTG GTG AAG TG
	F3 (forward)	C GAGCTC G TTGACAATTA ATC ATC GGC TCGTATAATG GATCG AAT TGT GAG
	R3 (reverse) (overlapping region of *csYF-citC*)	GCTTTTGTATCAGCCAT AAT CTA TGG TCC TTG TTG GTG AAG TG
pqq	F2 (forward)	CAAGGA CCATAGATT ATG GCC TGG AAC ACA CCG A
	R2 (reverse)	CC CTCGAG GG TTA CGT ATA ACG CCT GTA GAA CAA CGT GC
csYF-citC (artificial citrate operon)	F4 (forward)	CAAGGACCATAGATT ATG GCT GAT ACA AAA GC A AAA CTC ACC CTC
	R4 (reverse)	C GAGCTC G TTA CAC CAT CAT GCT GAA CAC GAT GC

pqq, pyrroloquinoline quinone

to considerable extent, were seen by alloying chelators like monoisoamyl DMSA (meso 2,3-dimercaptosuccinic acid) and succimer with antioxidants like taurine, melatonin, lipoic acid, *N*-acetylcysteine, and gossypin.

The gastrointestinal microbiota prevents absorption of ingested metal and allows only 40% to 60% of the metal to be transported across the intestinal barrier by binding and sequestering them on their cell membranes [17–19]. Probiotic *Escherichia coli* Nissle 1917 (*Ec*N) is available as licensed drug MutaflorR in Germany and other European countries. *Ec*N is noninvasive and nonpathogenic; does not produce enterotoxins or cytotoxins; and has been found to be therapeutically effective against ulcerative colitis, chronic constipation, and diarrhea [20]. *Ec*N also is known to impede reactive oxygen species [21]. The probiotic *E. coli* CFR 16 expressing the *Vitreoscilla* hemoglobin (*vgb*) and *pqq* genes not only acts as an antioxidant but protects against CCl_4 and dimethyl hydrazine–induced liver and colon damage and also altered neurotransmitter status [22–24]. Similarly, *Ec*N-4 and *Ec*N-5 producing PQQ was found to be more effective than orally administered PQQ against alcohol- and rotenone-induced oxidative stress [25,26].

Most bacteria, including gut microbiota, do not secrete citric acid. However, high levels of citric acid secretion up to 9 mM was achieved in many bacteria by overexpression of an artificial operon (*csYF-citC*) consisting of NADH-insensitive *E. coli cs* Y146 F mutant gene along with *S. typhimurium* Na^+-dependent citrate transporter (*citC*) gene [27–29]. The aim of the present study was to evaluate the effect of *Ec*N-20 producing PQQ with orally supplemented citric acid and *Ec*N-21 possessing artificial citrate operon (*csYF-citC*) and *pqq* gene cluster producing citric acid along with PQQ as a combination therapy against Cd- and Hg-induced hepatotoxicity and nephrotoxicity.

Methods and materials

Animals

Male adult Charles foster albino rats (weight 250–300 g) were given free access to food and water and were maintained at relative humidity (45.5%), controlled temperature (25°C ± 1°C), and photoperiod cycle (12-h light/12-h dark) according to the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) guidelines of Animal Ethical Committee (M. S. University of Baroda, India, Reg. No. 938/A/06/CPCSEA).

Cloning

Constitutive *tac* promoter (p*tac*) was amplified from pMALp2 using F1 and R1 primers (Tables 1 and 2). *pqq* gene cluster was amplified from the genome of *Gluconobacter oxydans* using Forward primer (F2) and Reverse primer (R2) to obtain amplicon of 3.3 kbp. Amplicons of p*tac* and *pqq* gene cluster have common overlapping regions of 24 bps. These two amplicons were used for recombinant polymerase chain reaction (PCR) with F1 and R2 primers to generate p*tac-pqq* amplicon of 3.4 kb, which was then cloned into pJET to get pRN1. Artificial citrate operon (*csYF-citC*) of 3.1 kb was amplified from pJNK4 using Forward primer F4 and Reverse primer R4. Constitutive p*tac* amplicon was obtained from pMALp2 using Forward primer F3 and Reverse primer R3. Constitutive p*tac* and *csYF-citC* amplicons have overlapping regions of 32 bps. Recombinant PCR of these two amplicons was performed using Forward primer F3 and Reverse primer R4 amplified p*tac-csYF-citC* of 3.2 kb, which was then cloned in pJET to get pRN2 plasmid. pRN1 plasmid was then digested with XhoI to release p*tac-pqq*, which was ligated with XhoI-digested linear pRN2 plasmid to get pRN3 plasmid. *Ec*N-20 was obtained by transforming pRN1 into *Ec*N-2 and *Ec*N-21 by transforming pRN3 into *Ec*N-2. *Ec*N was obtained from Dr. Rer. Nat. Ulrich Sonnenborn, Ardeypharm GmbH, Herdecke (Germany). *Ec*N strain was modified with genomic integration of *vgb* and *gfp* genes to produce *Ec*N-2 [26].

Characterization of Ec*N transformants producing PQQ and citric acid*

*Ec*N-20 and *Ec*N-21 transformants were confirmed by growing in Tris-buffered medium with methyl orange as pH indicator, appearance of red color indicated a pH drop due to gluconic acid secretion mediated by PQQ-dependent glucose dehydrogenase [30]. Extraction of PQQ from *Ec*N transformants was

Table 2
Plasmids and bacterial strains

Plasmids/Strain	Characteristics	References
pMALp2	P*tac, malE*, Amp^r	New England Biolabs
pJNK4	pUCPM18 with *Escherichia coli* NADH-insensitive citrate synthase gene (*csYF*), citrate transporter *citC* of *Salmonella typhimurium*; Apr, Kmr	Wagh et al., 2014 [28]
pJET	pJET1.2/blunt is a linearized cloning vector, recircularized pJET expresses a lethal restriction enzyme after transformation and is not propagated, Amp^r	Thermo Scientific CloneJET PCR Cloning Kit #K1231, #K1232
pRN1	pJET harboring gluconobacter oxydens *pqq* gene cluster (3.3 Kb) under *tac* promoter, Amp^r	This study
pRN2	pJET harboring *csYF-citC* genes from pJNK4 under *tac* promoter, Amp^r	This study
pRN3	pJET harboring gluconobacter oxydens *pqq* gene cluster under *tac* promoter and *csYF-citC* genes from pJNK4 under *tac* promoter	This study
*Ec*N	*Ec*N 1917 (probiotic strain)	Sonnenborn et al., 2009 [20]
*Ec*N-2	*Ec*N strain with genomic integration of *vgb* and *gfp* genes	Singh et al., 2014 [26]
*Ec*N-20	*Ec*N-2 harboring pJET-*tac-pqq* producing PQQ	This study
*Ec*N-21	*Ec*N-2 harboring pJET-*tac-pqq* and pJET-*tac-csYF-citC* producing PQQ and citric acid	This study

*Ec*N, *Escherichia coli* Nissle; PQQ, pyrroloquinoline quinone

Table 3
Experimental design for animal experiment

Group 1: Drinking water as control group*			
Group 2	100 ppm Cd in drinking water daily for 4 wk	Group 10	80 ppm Hg in drinking water daily for 4 wk
Group 3	Cd and 10^8 colony-forming units of *Ec*N-2/rat for 3 consecutive days following streptomycin wash (5 g/L for 24 h) dissolved in sterile normal saline once per week	Group 11	Hg and *Ec*N-2 once per week
Group 4	*EcN-2*, Cd, and citric acid (1.2 g/kg body weight) orally once a week	Group 12	*Ec*N-2, Hg, and citric acid (1.2 g/kg body weight) orally once a week
Group 5	*EcN-2*, Cd, and PQQ (10 mg/kg body weight) orally once a week	Group 13	*Ec*N-2, Hg, and PQQ (10 mg/kg body weight) orally once a week
Group 6	*EcN-20* and Cd	Group 14	*Ec*N-20 and Hg
Group 7	*EcN-21* and Cd	Group 15	*Ec*N-21 and Hg
Group 8	*EcN-2*, Cd, PQQ (10 mg/kg body weight orally), and citric acid (1.2 g/kg body weight orally) once a week	Group 16	*Ec*N-2, Hg, PQQ (10 mg/kg body weight orally), and citric acid (1.2 g/kg body weight orally) once a week
Group 9	*EcN-20*, Cd, and citric acid (1.2 g/kg body weight orally) once a week	Group 17	*Ec*N-20, Hg, and citric acid (1.2 g/kg body weight) orally once a week

Cd, cadmium; *Ec*N, *Escherichia coli* Nissle; Hg, mercury; PQQ, pyrroloquinoline quinone
* Group 1 as control group is common to Cd- and Hg-treated groups.

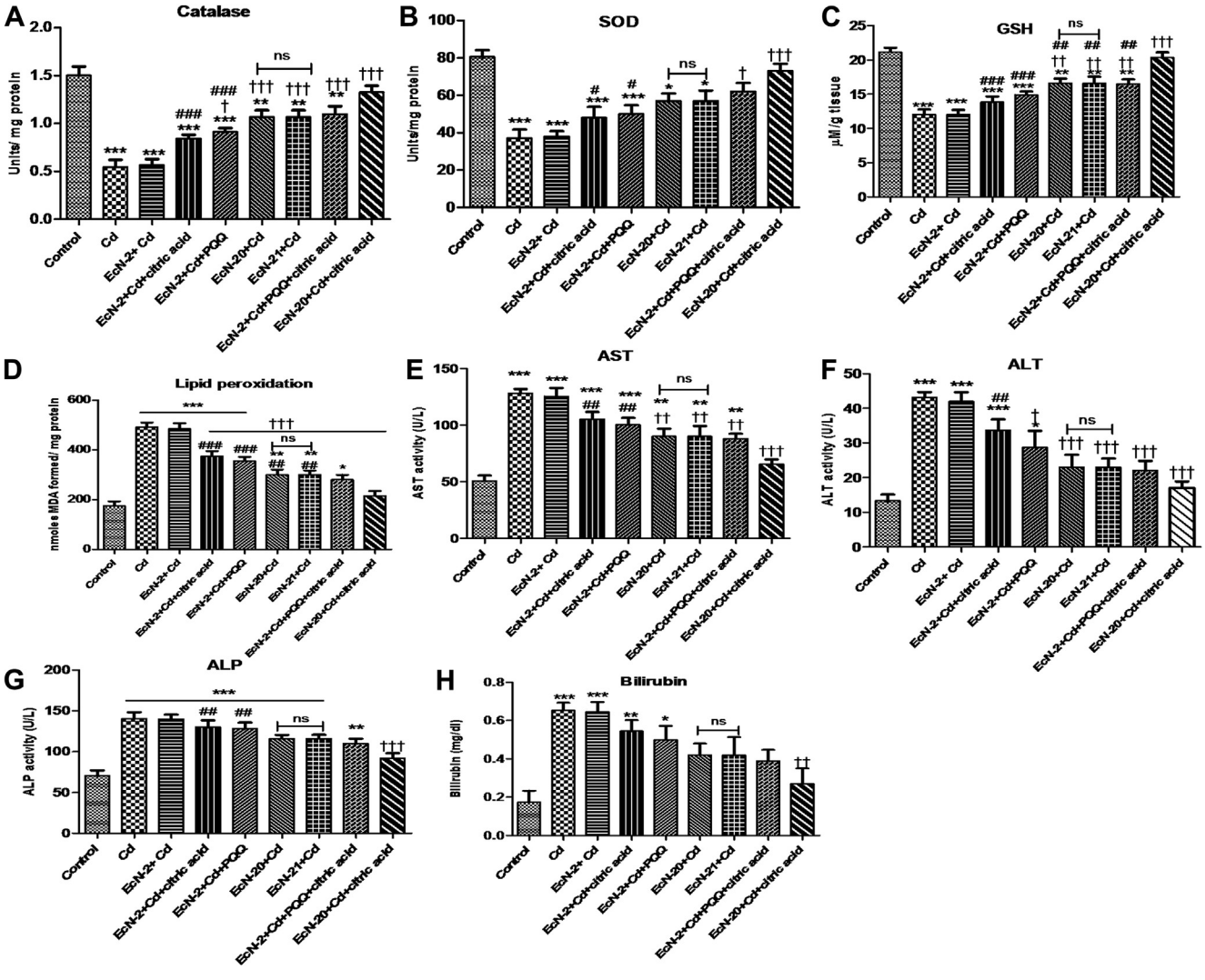

Fig. 1. Effects of genetically engineered probiotic *Escherichia coli* Nissle (*Ec*N) 1917 in liver on Cd exposure: (A) catalase, (B) SOD activity, (C) GSH levels, (D) lipid peroxidation, (E) AST, (F) ALT, (G) ALP activity, and (H) bilirubin levels in serum. Values are expressed as mean ± SEM (n = 6 each group). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ compared with control group; $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, and $^{\dagger\dagger\dagger}P \leq 0.001$ compared with Cd group; $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, and $^{\#\#\#}P \leq 0.001$ compared with *Ec*N-20 + citric acid (oral) + Cd. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Cd, cadmium; GSH, glutathione; PQQ, pyrroloquinoline quinone; SOD, superoxide dismutase.

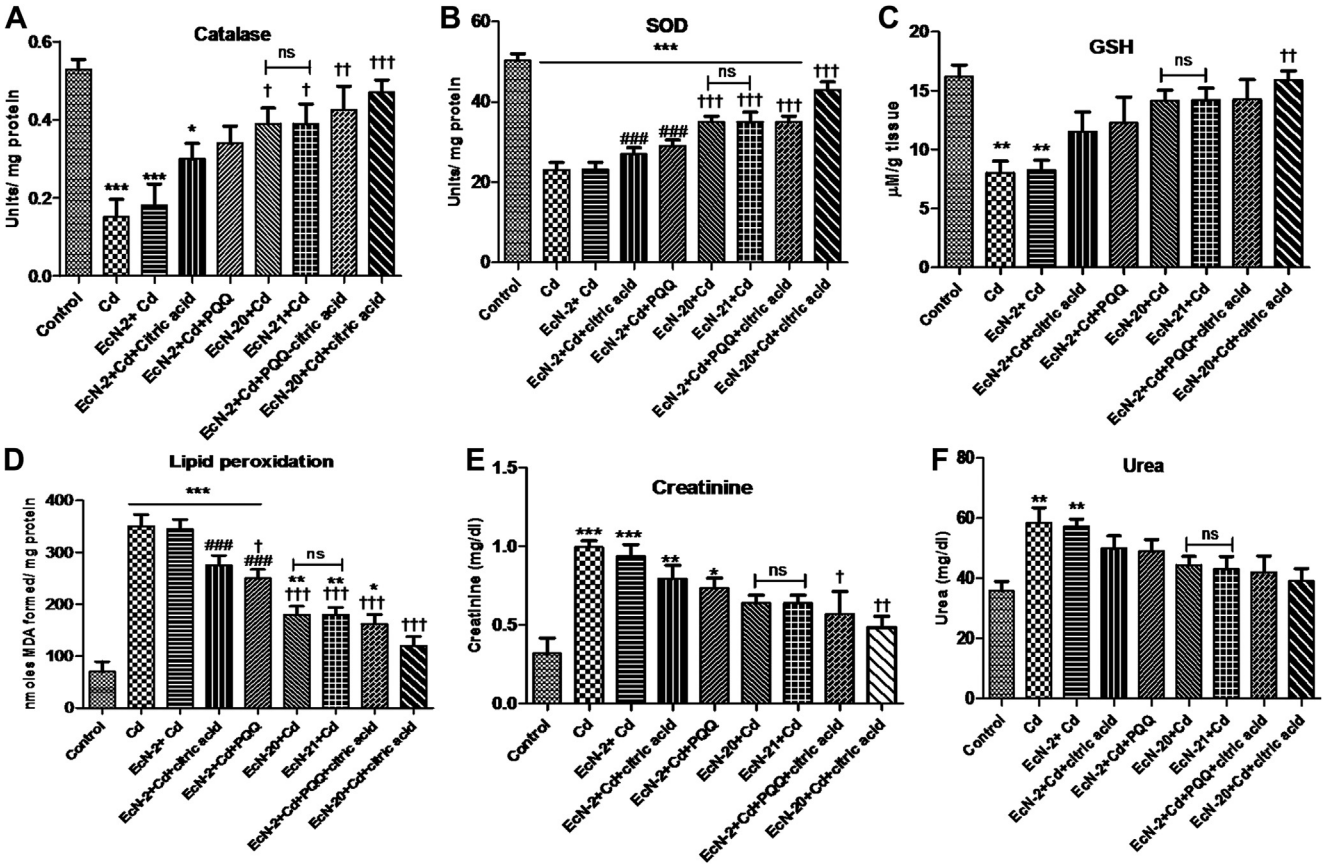

Fig. 2. Effects of genetically engineered probiotic *Escherichia coli* Nissle (*Ec*N) 1917 in kidney on Cd exposure: (A) catalase, (B) SOD activity, (C) GSH, (D) lipid peroxidation, (E) creatinine, (F) urea levels in serum. Values are expressed as mean ± SEM (n = 6 each group). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ compared with control group; $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, and $^{\dagger\dagger\dagger}P \leq 0.001$ compared with Cd group; $^{\#\#\#}P \leq 0.001$ compared with *Ec*N-20 + citric acid (oral) + Cd. Cd, cadmium; GSH, glutathione; MDA, malondialdehyde; PQQ, pyrroloquinoline quinone; SOD, superoxide dismutase.

performed according to a previously described method [31] and quantified using Hitachi fluorescence spectrophotometer (Hitachi High-Technologies Corporation, Tokyo, Japan) with excitation 375 nm and emission 465 nm. PQQ concentration of unknown sample was determined by plotting standard curve using PQQ standard. We used 20% liver tissue homogenate and 10% colonic content homogenate in phosphate buffer saline (PBS) for PQQ extraction and quantification.

Citric acid excreted by *Ec*N transformants was determined by high-performance liquid chromatography (HPLC) analysis according to a previously described method [32]. For HPLC analysis, the RP-18 column was operated at room temperature using 0.02% orthophosphoric acid as mobile phase, at a flow rate of 0.8 mL/min and the column effluents were monitored using an ultraviolet detector at 210 nm. Quantification was done by using peak areas obtained for the citric acid standard (Sigma Pvt. Ltd., India).

Bacterial strains and culture conditions

Probiotic *Ec*N-2, *Ec*N-20, and *Ec*N-21 strains were grown in Luria Broth (LB) overnight at 37°C followed by reinoculation in fresh medium to achieve final colony-forming unit (CFU) of 10^9 cells/mL culture. One mL of this culture was pellet down and washed twice with saline followed by redissolving pellet into saline and then tube fed to different rat groups receiving probiotic dose.

Experimental design

All rats were fed a normal pellet diet and were divided into17 different groups (six animals per group) as shown in Table 3.

Preparation of tissue homogenates

Liver and kidney tissues were washed with ice-cold saline after sacrificing rats, then homogenization was performed in ice-cold PBS. To estimate glutathione (GSH) levels, tissues were homogenized separately in 5% trichloroacetic acid.

Biochemical assays

Catalase activity was determined by a previous protocol [33]. Superoxide dismutase (SOD) activity was determined by a previous method [34]. Reduced GSH was determined by a previously described method [35]. By estimating levels of malondialdehyde (MDA), lipid peroxidation was measured according to a previously described method [36].

ALT, AST, ALP, total bilirubin, urea, and creatinine

Alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), total bilirubin, urea, and creatinine in serum were measured using kit as per manufacturer protocol (Beacon Diagnostics Pvt. Ltd., Navsari, Gujarat, India).

Histopathologic changes

Liver and kidney tissue were fixed in 10% buffered formalin, dehydrated in ascending grades of ethyl alcohol, cleared in xylene, and mounted in molten paraplast 58°C to 62°C. 5 µm histologic sections were cut and stained with hematoxylin and eosin and examined under bright field light microscope.

Metal determination

Estimation of Cd and Hg was done from colonic contents by atomic absorption spectroscopy according to a previous protocol [37]. Cd and Hg content were expressed in mg/g of rat feces.

Statistical analysis

The statistical significance of the values was determined by one-way analysis of variance (ANOVA) using GraphPad Prism Version 5.0 (GraphPad

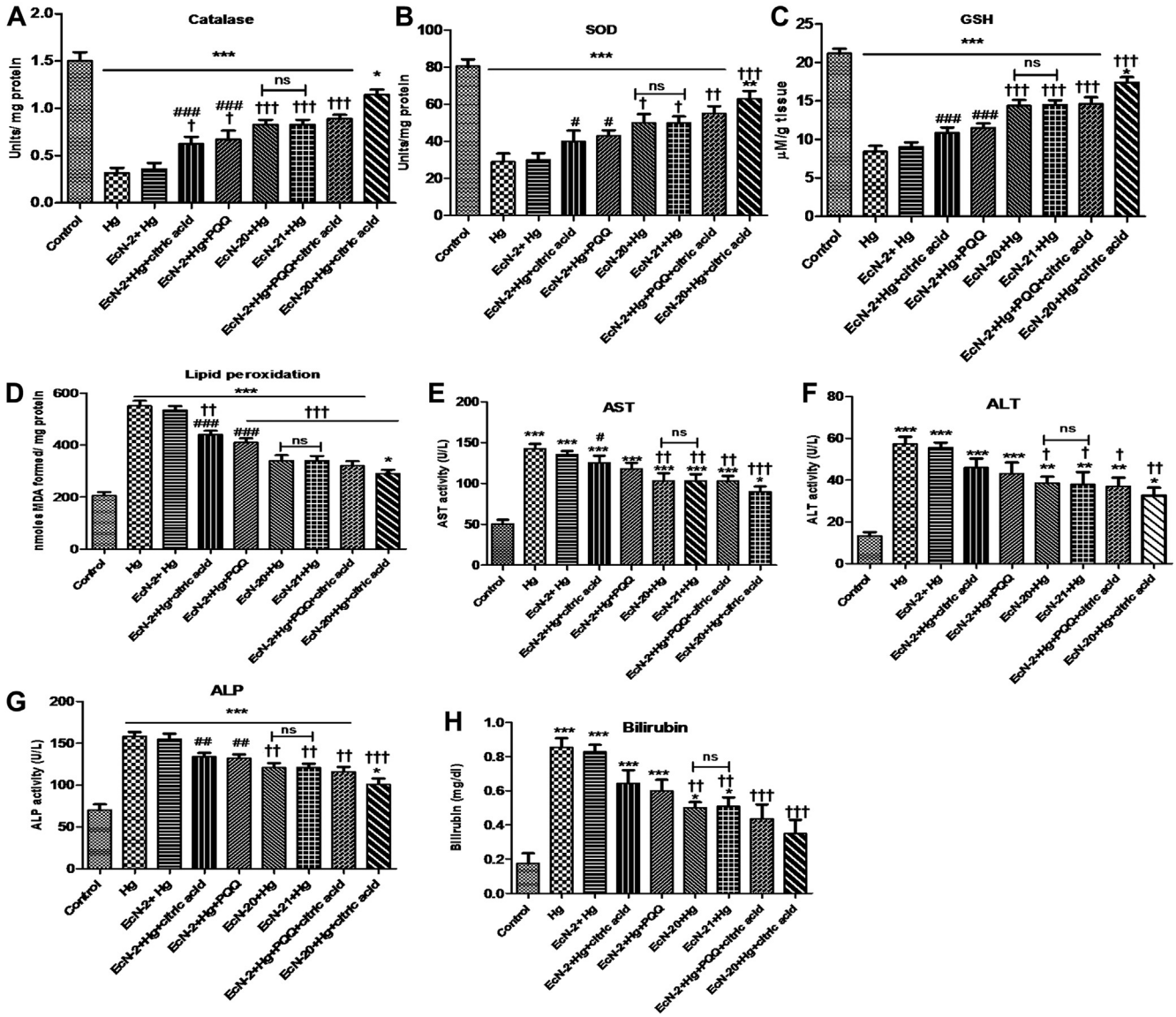

Fig. 3. Effects of genetically engineered probiotic *Escherichia coli* Nissle (*Ec*N) 1917 in liver on Hg exposure: (A) catalase, (B) SOD activity, (C) GSH levels, (D) lipid peroxidation, (E) AST, (F) ALT, (G) ALP activity, and (H) bilirubin levels in serum. Values are expressed as mean ± SEM (n = 6 each group). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ compared with control group; $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, and $^{\dagger\dagger\dagger}P \leq 0.001$ compared with Hg group; $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, and $^{\#\#\#}P \leq 0.001$ compared with *Ec*N-20 + citric acid (oral) + Hg. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Hg, mercury; GSH, glutathione; PQQ, pyrroloquinoline quinone; SOD, superoxide dismutase.

Softwares Inc., San Diego, CA, USA). The results were considered significant at $P \leq 0.05$.

Results

Characterization of Ec*N-2 transformants*

*Ec*N-20 and *Ec*N-21 transformants produced 5.6 ± 0.12 µg PQQ/mL of culture after 24 h in M9 minimal medium at 37°C with glucose as carbon source. Both transformants acidified tris-buffered medium with methyl red as an indicator, which was due to secretion of gluconic acid by PQQ-dependent glucose dehydrogenase. Additionally, *Ec*N-21 secreted 2 ± 0.09 mM of citric acid.

Effect of Ec*N-2 transformants against Cd- and Hg-induced liver and kidney damage in rats*

Cd and Hg exposure for 1 month decreased the GSH levels and catalase and SOD activity, while it increased the lipid peroxidation significantly in liver and kidney compared with control. They also increased the AST, ALT, and ALP activities and bilirubin, urea, and creatinine levels significantly in serum compared with control, whereas *Ec*N-2 treatment had no effect (Figs. 1–4). However, *Ec*N-20 + citric acid (oral) was most effective against Cd, as well as Hg exposure, by increasing GSH levels and catalase and SOD activity, in decreasing the lipid peroxidation in liver and kidney and in decreasing AST, ALT, and ALP activities and bilirubin, creatinine, and urea levels in serum compared with

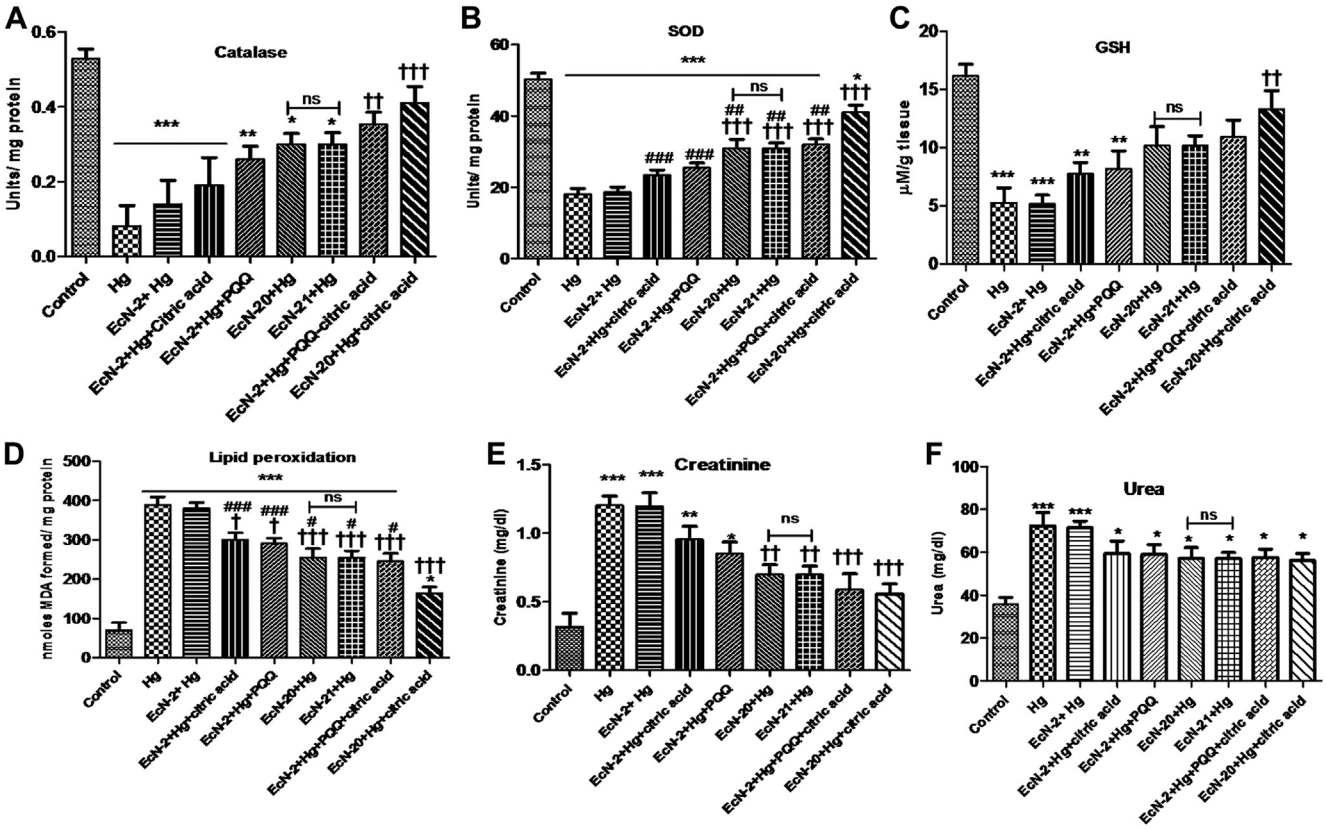

Fig. 4. Effects of genetically engineered probiotic *Escherichia coli* Nissle (*Ec*N) 1917 in kidney on Hg exposure: (A) catalase, (B) SOD activity, (C) GSH, (D) lipid peroxidation, (E) creatinine, (F) urea levels in serum. Values are expressed as mean ± SEM (n = 6 each group). $^{*}P \leq 0.05$, $^{**}P \leq 0.01$, and $^{***}P \leq 0.001$ compared with control group; $^{\dagger}P \leq 0.05$, $^{\dagger\dagger}P \leq 0.01$, and $^{\dagger\dagger\dagger}P \leq 0.001$ compared with Hg group; $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$, and $^{\#\#\#}P \leq 0.001$ compared with *Ec*N-20 + citric acid (oral) + Hg. GSH, glutathione; Hg, mercury; MDA, malondialdehyde; PQQ, pyrroloquinoline quinone; SOD, superoxide dismutase.

*Ec*N-2 + citric acid (oral), *Ec*N-2 + PQQ (oral), *Ec*N-20, *Ec*N-21, *Ec*N-2 + PQQ (oral) + citric acid (oral). Nevertheless, protection shown by *Ec*N-20 + citric acid (oral) was not sufficient in bringing the damage to normal levels against Hg toxicity, whereas in Cd toxicity it helped in restoring near-normal levels. For Cd and Hg toxicity, *Ec*N-20 was more effective than *Ec*N-2 + PQQ (oral) in increasing catalase and SOD activity and GSH levels and in decreasing lipid peroxidation in liver and kidney, also in decreasing AST, ALT, and ALP activities and bilirubin and creatinine levels in serum. Moreover, protection shown by *Ec*N-21 was found to be the same as that of *Ec*N-20.

PQQ quantification from feces and liver

PQQ levels were estimated from feces and liver of rats treated with *Ec*N-20 and *Ec*N-21 (Table 4). In Cd exposure, levels of PQQ were found to be 4.45 ± 0.2, 4.51 ± 1.16, 4.68 ± 0.19 nmol/g wet weight of feces and 235.51 ± 5.6, 224.64 ± 8.1, 250.2 ± 8.3 picomol/g liver tissue in groups 6, 7, and 9, which were significantly higher compared with the control group and PQQ (oral) group (group 5), whereas in Hg exposure, PQQ levels were found to be 4.65 ± 0.24, 4.34 ± 0.21, 4.92 ± 0.26 nmol/g wet weight of feces, and 232.15 ± 7.7, 220.84 ± 6.4, 242.62 ± 3.9 picomol/g liver

Table 4
PQQ concentration in fecal matter and liver homogenate of rats

Cd groups	1	2	3	4	5	6	7	8	9
Fecal (nmol/g fecalwet weight)	0.697 ± 0.12	0.709 ± 0.19	0.728 ± 0.13	0.717 ± 0.23	0.779 ± 0.15	4.45 ± 0.2***	4.51 ± 1.16***	0.764 ± 0.23	4.68 ± 0.19***
Liver (picomol/g tissue)	23.16 ± 7.1	25.12 ± 4.1	24.64 ± 3.4	26.51 ± 5.1	30.11 ± 7.8	235.51 ± 5.6***	224.64 ± 8.1***	31.05 ± 5.4	250.20 ± 8.3***
Hg groups	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	
Fecal (nmol/g fecal wet weight)	0.681 ± 0.16	0.705 ± 0.09	0.728 ± 0.11	0.785 ± 0.22	4.65 ± 0.24†††	4.34 ± 0.21†††	0.773 ± 0.28	4.92 ± 0.26†††	
Liver (picomol/g tissue)	24.61 ± 5.4	25.12 ± 6.7	23.89 ± 7.2	30.74 ± 9.2	232.15 ± 7.7†††	220.84 ± 6.4†††	30.91 ± 7.1	242.62 ± 3.9†††	

Cd, cadmium; Hg, mercury
*** $P \leq 0.001$ compared with groups 1 and 5.
††† $P \leq 0.001$ compared with groups 1 and 13.

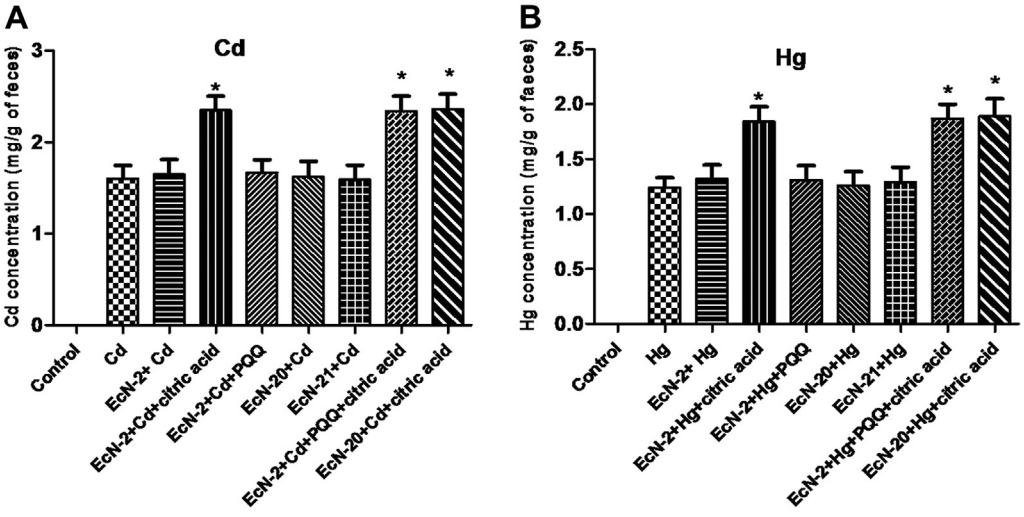

Fig. 5. Effects of genetically engineered probiotic *Escherichia coli* Nissle (*Ec*N) 1917 on levels of (A) Cd and (B) Hg in feces of rats. Values are expressed as mean ± SEM (n = 6 each group). $^{*}P \leq 0.05$ compared with Cd/Hg, *Ec*N-2 + Cd/Hg, *Ec*N-2 + Cd/Hg + PQQ (oral), *Ec*N-20 + Cd/Hg, *Ec*N-21 + Cd/Hg. Cd, cadmium; Hg, mercury; PQQ, pyrroloquinoline quinone.

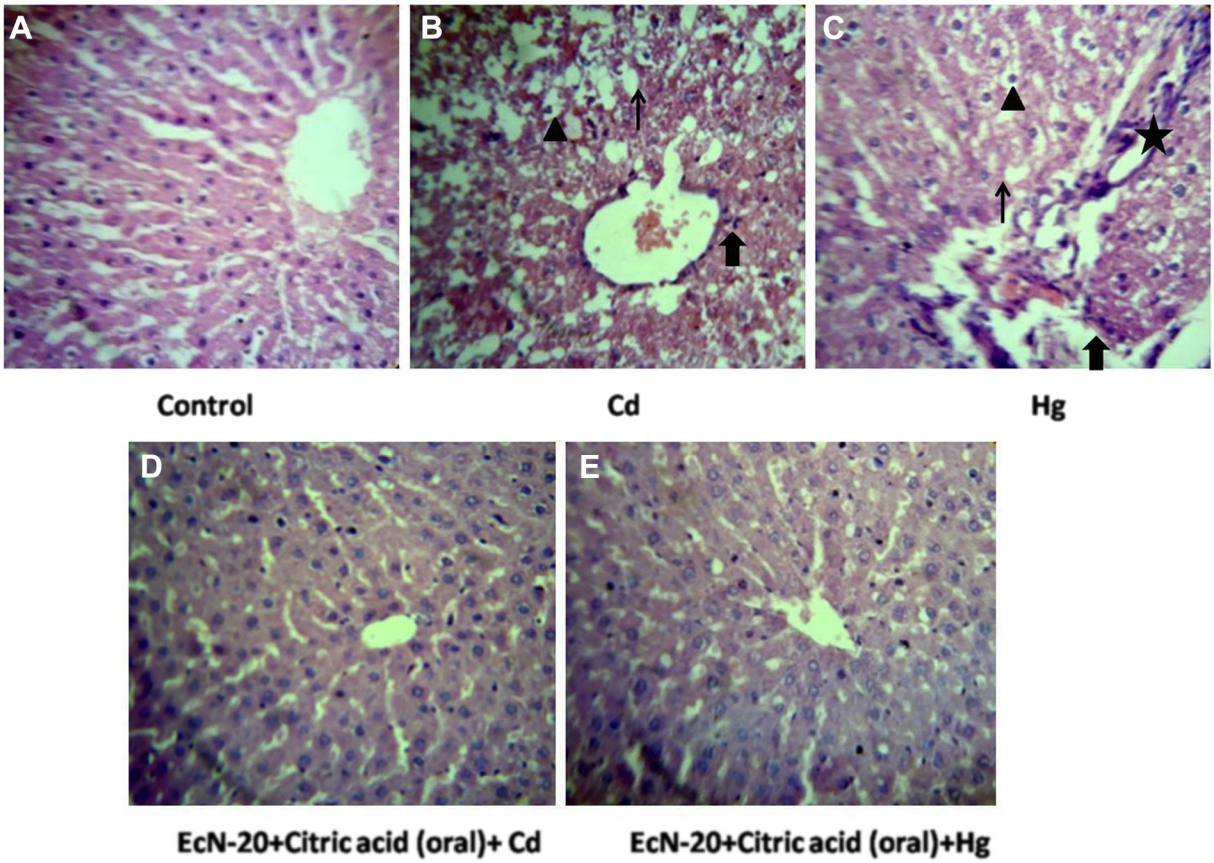

Fig. 6. Photomicrograph of liver stained with HE (magnification = 40×). (A) Control showing normal liver architecture. (B) Cd-treated group showing hepatocyte vacuolation (black arrow), perinuclear halo (arrow head), necrosis (bold arrow). (C) Hg-treated group showing hepatocyte vacuolation (black arrow), perinuclear halo (arrow head), necrosis (bold arrow), inflammatory cell infiltration, and sinusoidal dilation (star). (D) *Ec*N-20 + Cd + citric acid (oral) and (E) *Ec*N-20 + Hg + citric acid (oral) showing near to normal appearance of liver. Cd, cadmium; *Ec*N, *Escherichia coli* Nissle; Hg, mercury.

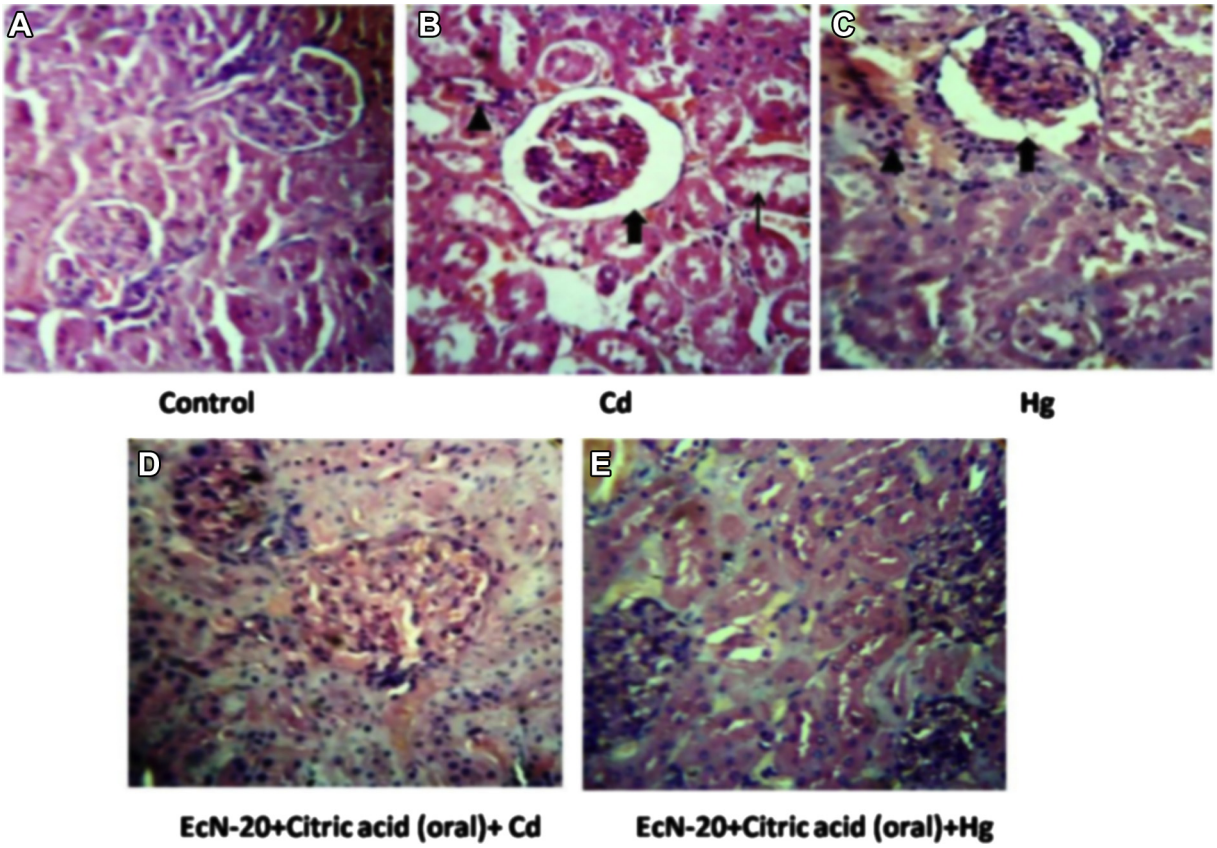

Fig. 7. Photomicrograph of kidney stained with HE (magnification = 40×). (A) Control showing normal kidney architecture. (B) Cd-treated group showing renal tubular dilation (black arrow), renal tubular necrosis (arrow head), shrinkage of glomeruli, and distention of bowman's capsule (bold arrow). (C) Hg-treated group showing renal tubular necrosis (arrow head), shrinkage of glomeruli, and distention of bowman's capsule (bold arrow). (D) *Ec*N-20 + Cd + citric acid (oral) and (E) *Ec*N-20 + Hg + citric acid (oral) showing moderate improvement of renal tubules, with detected glomerulonephritis having missing mesangial space. Cd, cadmium; *Ec*N, *Escherichia coli* Nissle; Hg, mercury.

tissue in groups 14, 15, and 17, which were significantly higher compared with the control and PQQ (oral) group (group 13).

Cd and Hg estimation

Cd and Hg were estimated from fecal matter by atomic absorption spectroscopy (Fig. 5). Fecal Cd levels were significantly higher in groups 4, 8, and 9 with orally administered citric acid compared with Cd, *Ec*N-2 + Cd, *Ec*N-2 + Cd + PQQ (oral), *Ec*N-20 + Cd, and *Ec*N-21 + Cd. Likewise, fecal Hg levels were significantly higher in groups 12, 16, and 17 with orally administered citric acid compared with Hg, *Ec*N-2 + Hg, *Ec*N-2 + Hg + PQQ (oral), *Ec*N-20 + Hg, and *Ec*N-21 + Hg.

Histologic analysis

Histologic analysis showed that the control group had normal architecture, whereas introduction of Cd and Hg induced pathologic changes in liver and kidney (Figs. 6 and 7). Cd and Hg exposure caused hepatocyte vacuolation, perinuclear halo, necrosis, inflammatory cell infiltration and sinusoidal dilation, and derangement of hepatic cords, whereas in kidneys it leads to renal tubular dilation, necrotic renal tubules, distention of Bowmen's cavity, and shrinkage and reduction of glomeruli. Treatment with *Ec*N-20 + citric acid (oral) caused near to normal appearance in liver, whereas in the kidney there was a moderate improvement of renal tubules with detected glomerulonephritis having missing mesangial space.

Discussion

Major sources of exposure of heavy metals like Cd and Hg are contaminated food and water [2]. Liver and kidney are mainly sensitive to Cd and Hg, with damage proportionate to the quantity of Cd and Hg not bound to metallothionein (MT). MT forms complex with heavy metals and decreases their body burden. Once MT gets saturated, unbound metal forms increase and cause subsequent damage [38]. Oxidative stress is a result of higher reactivity of heavy metals for sulfhydryl groups located at the active site of antioxidant enzymes as well as with tripeptide GSH, thus decreasing their levels [39,40]. Antioxidant enzymes can be inactivated by heavy metals by replacing cofactor of these enzymes [41]. From ceruloplasmin and ferritin, iron and copper can be replaced by Cd, thus generating free radicals by the Fenton reactions by increasing the total volume of free iron and copper ions [42]. Animal studies have suggested that Cd and Hg altered the activities of antioxidant enzymes as well as GSH and MDA levels in liver and kidney [43–45]. Glomerular filtration has been shown to be reduced by tubular injury induced by reactive oxygen species, thus ultimately increasing urea and creatinine levels in blood [46]. Elevated levels of AST, ALT, ALP, and bilirubin are the markers for acute liver damage [47,48]. The results of the

present study demonstrated significant damage to liver and kidney due to Cd and Hg.

Previously, the presence of 10^{14} bacterial cells in gut was suggested to confer several resistance mechanisms such as by sequestering metals or by actively transporting metal out of the cell as well as by transforming them to a less toxic form [49–51]. Interestingly, *Lactobacillus plantarum* CCFM8610 treatment was found to prevent renal and hepatic oxidative stress, reduce tissue Cd accumulation, and also improved hepatic histopathology [52]. Although *Ec*N-2 was not effective in dealing with toxicity associated with Cd and Hg, *Ec*N-20 was found to be more effective than *Ec*N-2 with PQQ (oral). Likewise, similar results were shown by probiotic *E. coli* CFR 16:v*gb-gfp* (*pqq*) and *Ec*N secreting PQQ against oxidative stress induced by administration of DMH, alcohol, and rotenone [23,25,26].

Interestingly, the present study demonstrated that *Ec*N-20 with citric acid (oral) was most effective in dealing metal toxicity. Most widely used clinically important chelators include EDTA, dimercaptopropane sulfonate (DMPS), dimercaptosuccinic acid (DMSA), and British Anti-Lewisite (BAL) [53]. Chelators have been proposed to mobilize heavy metals for excretion from kidney in urine and liver through bile [54]. Protection shown by *Ec*N-20 + citric acid (oral) was probably due to constitutive production of PQQ within gut by *Ec*N-20, PQQ then absorbed by gut reaches to different organs like liver and kidney, where it scavenges free radicals produced by Cd and Hg. Additionally, the supplementation of citric acid will not only chelate Cd and Hg in gut lumen but also in liver and kidney facilitating excretion of Cd and Hg. Citric acid is known to enhance biliary excretion of metals [55]. Both *Ec*N-20 and *Ec*N-21 produce PQQ which, acting as cofactor for glucose dehydrogenase, facilitates the conversion of glucose to gluconic acid. Gluconic acid is known to act as prebiotic [56] and its use by beneficial microbiota results in the production of short-chain fatty acids (SCFAs), which in turn provide anti-inflammatory effects [57] and could prevent the proinflammatory effects of Cd and Hg. This is supported by results from a previous study that showed that fecal matter from rats treated with *Ec*N secreting PQQ had high levels of SCFAs like butyric and propionic acids [25]. Histopathologic studies have shown that the present strategy is more effective for liver than kidney because kidney is major organ affected by heavy metal toxicity [58]. *Ec*N-21 producing PQQ and citric acid was as effective as *Ec*N-20 producing PQQ alone. This could be attributed to inefficient chelation of Cd and Hg due to low levels of citric acid. Thus, the present strategy has moderate protective effects on Cd and Hg toxicity and paves the way for improvising the probiotics for better protection.

Conclusion

The present study demonstrates that *Ec*N-20 producing PQQ supplemented with citric acid orally is an effective strategy to deal with Cd- and Hg-induced liver and kidney damage compared with orally given PQQ and citric acid. This strategy was found to be more effective for Cd toxicity and protection was better in liver compared with kidney. Less effectiveness of *Ec*N-21 could be attributed to low levels of secreted citric acid.

Acknowledgments

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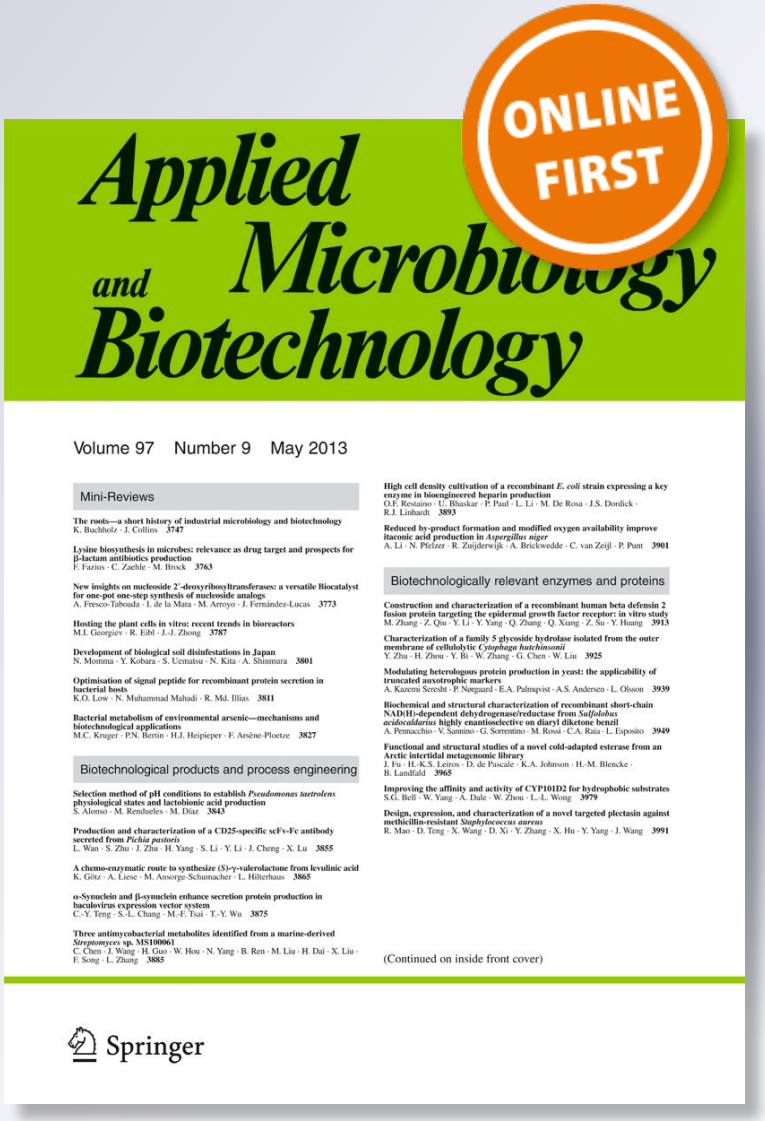

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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Genetically engineered *Escherichia coli* Nissle 1917 synbiotic counters fructose-induced metabolic syndrome and iron deficiency

Archana Somabhai Chaudhari[1] · **Ruma Raghuvanshi**[1] · **G. Naresh Kumar**[1]

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Abstract Consumption of fructose leads to metabolic syndrome, but it is also known to increase iron absorption. Present study investigates the effect of genetically modified *Escherichia coli* Nissle 1917 (*Ec*N) synbiotic along with fructose on non-heme iron absorption. Charles foster rats weighing 150–200 g were fed with iron-deficient diet for 2 months. Probiotic treatment of *Ec*N (*pqq*) and *Ec*N (*pqq-glf-mtl*K) was given once per week, 10^9 cells after 2 months with fructose in drinking water. Iron levels, blood, and liver parameters for oxidative stress, hyperglycemia, and dyslipidemia were estimated. Transferrin-bound iron levels in the blood decreased significantly after 10 weeks of giving iron-deficient diet. Probiotic treatment of *Ec*N (*pqq-glf-mtl*K) and fructose together led to the restoration of normal transferrin-bound iron levels and blood and hepatic antioxidant levels as compared to iron-deficient control group. The probiotic also led to the restoration of body weight along with levels of serum and hepatic lipid, blood glucose, and antioxidant in the blood and liver as compared to iron-deficient control group. Restoration of liver injury marker enzymes was also seen. Administration of *Ec*N-producing PQQ and mannitol dehydrogenase enzyme together with fructose led to increase in the transferrin-bound iron levels in the blood and amelioration of consequences of metabolic syndrome caused due to fructose consumption.

Keywords *E. coli* Nissle 1917 · Iron · Fructose · SCFA · Synbiotic

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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 and Rossander-Hulten 1991; Hallberg et al. 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 and Hulthén 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 (Christides and Sharp 2013). In Western diets, fruit is not the major source of fructose, but mainly 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-heme iron is enhanced by lactic acid-fermented foods in humans probably because of their ability to activate phytases and lower pH due to organic acid 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. 2000; Hoppe et al. 2015). Fermentation of almond milk with potentially probiotic bacteria enhanced the uptake of iron by Caco-2 cells, especially with *L. rhamnosus* and either *Bifidobacterium bifidum* or *B. longum* (Bernat et al. 2015).

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Prebiotics including fructooligosaccharide, inulin, mannitol, and 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 (SCFAs) that also help in iron absorption because they lower the pH of colon and thus help in absorption by releasing iron from complexes (Perez-Conesa et al. 2007). Consumption of *B. 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, whereby 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 dehydrogenase 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.

Material and methods

Bacterial strains and plasmids

Probiotic *Ec*N (DSM 6601) was acquired as a gift from Dr. Ulrich Sonnenborn (Ardeypharm GmbH, Loerfeldstrabe 20, Herdecke, Germany). The bacterial strains and plasmid constructs used in the current study are listed in Table 1. Probiotics were supplemented to different rat groups as per the procedure described by Singh et al. (2014).

Animals

For animal studies, adult albino male Charles Foster rats (180–200 g) were used. They were maintained in controlled condition as per 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 guidelines, i.e., temperature (25 ± 1 °C), relative humidity (45.5%), and photoperiod cycle (12-h light:dark) along with free access to water and food.

Designing of experiment

Charles Foster rats were divided initially into two different groups for the current study. Iron-deficient group was supplemented with AIN-93 iron-depleted diet for 10 weeks (n = 60), and the control group (n = 6) was fed with normal diet. Composition of the diet is shown in Table 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 groups 1 and 2 once per week till 1 month. The fecal plate count was done for confirmation of colonization of the probiotic. The iron-deficient group was then divided into 11 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 and iron. Group 5 received iron and ascorbic acid (150-mg/kg diet). Group 6 received iron and *Ec*N-2. Group 7 received fructose along with iron and *Ec*N-2. Group 8 received iron and *Ec*N (pAN1). Group 9 received fructose along with iron and *Ec*N (pAN1). Group 10 received iron and *Ec*N (pAN6). Group 11 received fructose along with iron and *Ec*N (pAN6).

Iron was given in the form of ferric citrate (0.2 g) in the diet, 20% fructose was given in the drinking water, and the amount of ascorbic acid given was 150 mg/kg of diet. At the end of the experiment, iron-depleted and control rats were fasted overnight and euthanized under ether anesthesia.

Biochemical estimation and assays

Enzymatic assays superoxide dismutase (SOD), catalase (CAT), lipid peroxidation, and reduced glutathione (GSH) were performed as described in Somabhai et al. (2016). SOD and CAT activities are reported as units per milligram protein. Liver function tests, lipid estimation, and iron content in blood plasma were estimated by using kits as per manufacturer protocol (Beacon Diagnostics Pvt. Ltd., Navsari, India).

Quantitative reverse transcription PCR and messenger RNA expressions

RNA extraction and cDNA synthesis were performed as described by Somabhai et al. (2016). PCR was performed for each sample in duplicate by ABI Quant-Studio TM 12-K flex real-time PCR system coupled with SYBR Green technology (Applied Biosystems) using primers CTTTGCAACTTCGT CGCTCC (forward) and GTCCTGGTGGTAGTTCTGGC (reverse) for ferritin-H (FH) and GATGAGTGGAGCGT CACGAG (forward) and CTGGCCTGCTATGTAGGCAT (reverse) for transferrin (Tf) gene.

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Table 1 Plasmid and bacterial strains used in the study

Plasmid/strains	Characteristics	Reference
Plasmids		
pJET vector	Cloning vector, PlacUV5, rep (pMB1), T7 promoter, Ap^r	Thermo Scientific CloneJET PCR Cloning Kit
pAN1	pJET vector containing p*tac**-*pqq*, Ap^r	Somabhai et al. (2016)
pAN6	pJET vector containing p*tac**-*pqq-glf-mtlK*, Ap^r	Somabhai et al. (2016)
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)	Somabhai et al. (2016)
*Ec*N (pAN6)	*Ec*N-2 containing *Ec*N (pAN6)	Somabhai et al. (2016)

Histopathological analysis

Histological sections of liver tissue were fixed with 10% buffered formalin and stained with hematoxylin and eosin stains. For analysis of sections, pathologist uninformed with experimental codes was consulted.

Statistical analysis

One-way ANOVA followed by Bonferroni comparisons was used for evaluation of differences in antioxidant enzymes, lipid peroxidation among six different groups. Results obtained by calculating using the commercially available statistical software packages (GraphPad Prism version 5.0; La Jolla, CA, 92037, USA) are expressed as means ± SEM.

Results

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. 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-deficient control.

Table 2 Composition of AIN-93 iron-depleted diet and normal diet

	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	0.212	000
Vitamin mix	10.0	10.0
Choline bitartrate	2.5	2.5

Source: Saini et al. (2014)

Serum profile

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. 2). So, it can be concluded that iron deficiency and not anemia has been induced.

Tf-bound iron levels in Fe-deficient control group are significantly lower, although the rats were given Fe in the diet. As compared to the Fe-deficient 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 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 did not show any significant changes in the fasting blood glucose levels (Fig. 1).

Both serum cholesterol and triglyceride levels 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). There was no significant difference in the cholesterol and triglyceride levels in the Fe-deficient control group as compared to the normal control group and the group fed with fructose and *Ec*N (pAN6).

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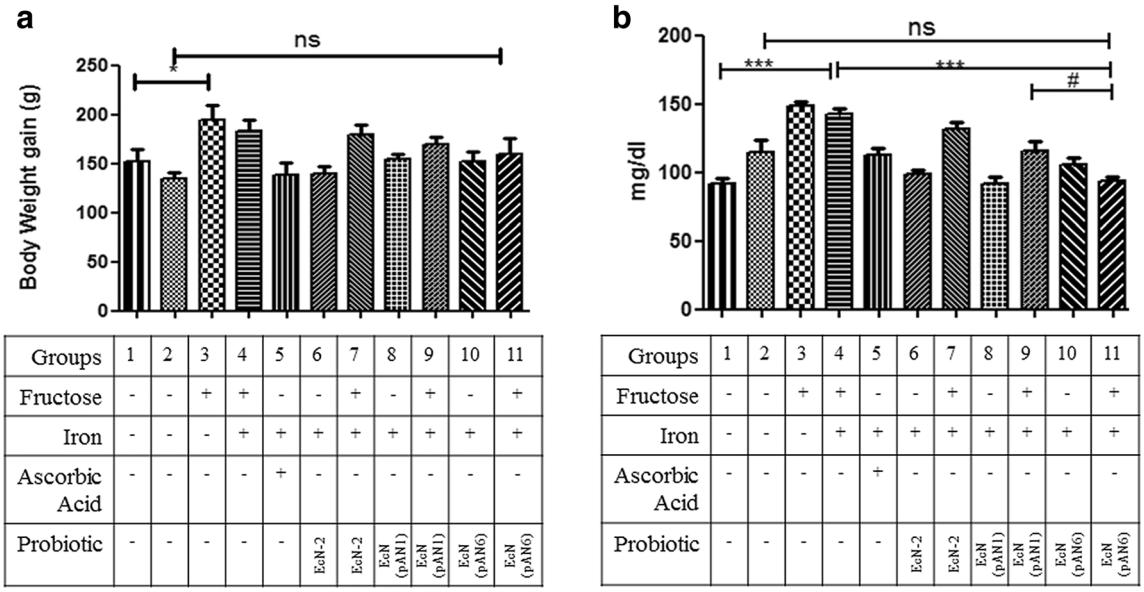

Fig. 1 Effect of genetically engineered probiotic *E. coli* Nissle 1917 on **a** body weight and **b** fasting blood glucose levels. Values are expressed as mean ± SEM (n = 5–6 each group). $^{*}P \leq 0.05$ compared with control group, $^{***}P \leq 0.001$ compared with control group, $^{\#}P \leq 0.05$ compared with fructose + iron + *Ec*N (pAN1)

Effect of probiotic treatment on antioxidant status

Extent of oxidative damage by fructose consumption in the liver and iron deficiency were determined using antioxidant parameters. Catalase and SOD activities 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-deficient control, which was found to be decreased in the *Ec*N (pAN1)- and *Ec*N (pAN6)-fed groups (Fig. 4). Reduced glutathione, which contributes in the reduction of oxidative stress in the body, was seen to be decreased in the fructose and Fe-deficient control

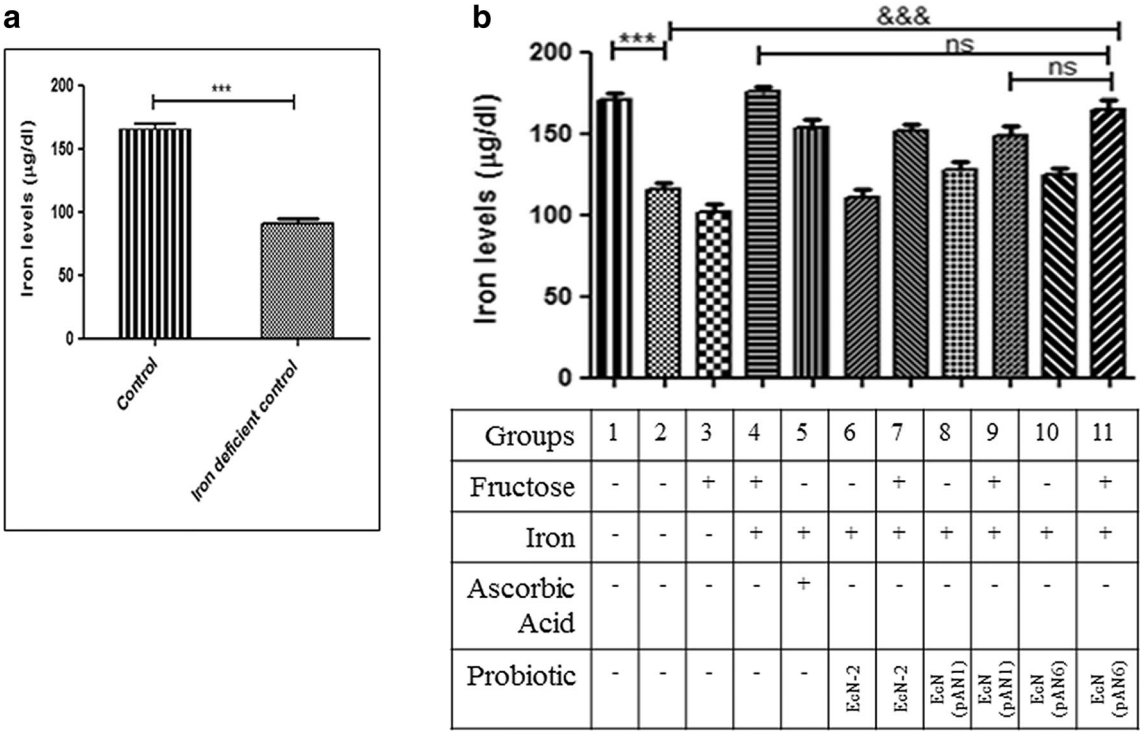

Fig. 2 Serum iron levels (**a**) before and (**b**) after the repletion period. Values are expressed as mean ± SEM (n = 6 each group). $^{***}P \leq 0.001$ compared with control, $^{\&\&\&}P \leq 0.001$ compared with iron-deficient control

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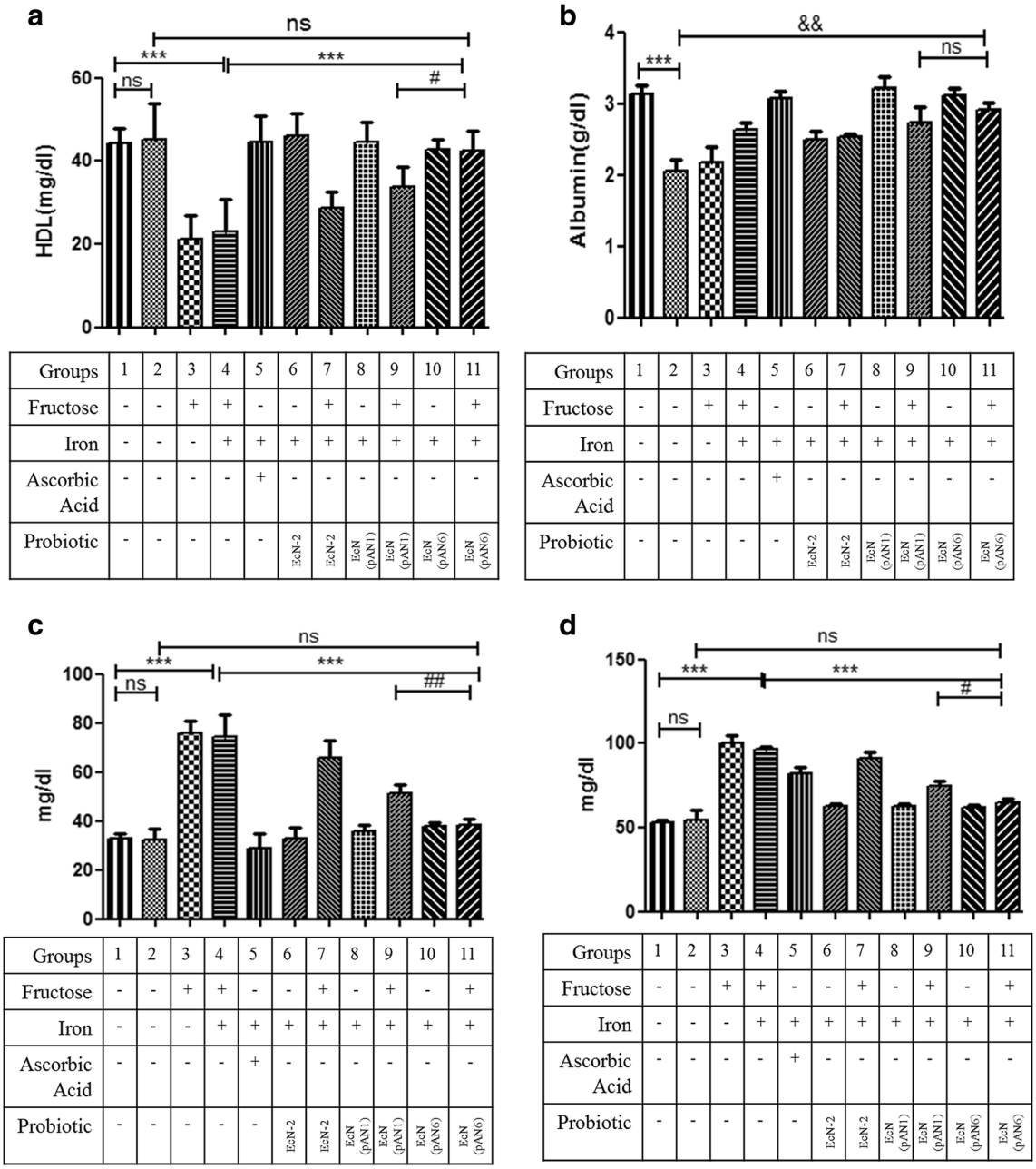

Fig. 3 Effect of genetically engineered probiotic *E. coli* Nissle 1917 on serum lipid profile **a** HDL, **b** albumin, **c** cholesterol, and **d** triglyceride levels. Values are expressed as mean ± SEM (n = 5–6 each group). $^{***}P \leq 0.001$ compared with fructose control, $^{\#}P \leq 0.05$, $^{\#\#}P \leq 0.01$ compared with fructose + iron + *Ec*N (pAN1)

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.

Similar results were obtained for the antioxidant status of blood as were obtained in the antioxidant status of liver. Catalase and SOD activities 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. 5). 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. Additionally, the lipid peroxidation was also reduced in probiotic-fed group.

Effect of probiotic treatment on liver function marker

Liver function markers, i.e., ALT and AST levels in the serum, were estimated to determine the oxidative stress in the liver due to iron deficiency and fructose consumption. AST and

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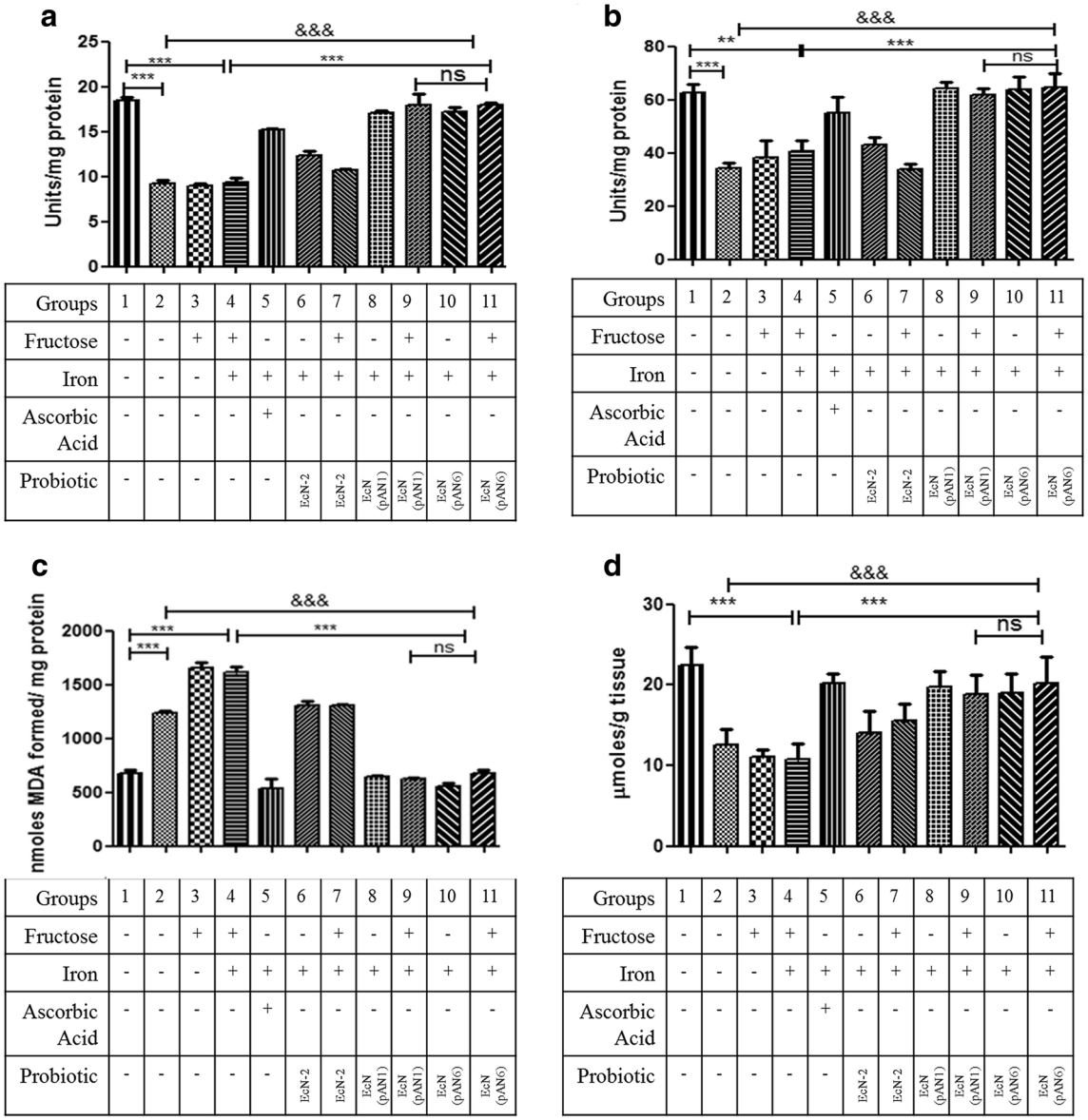

Fig. 4 Effect of genetically engineered probiotic *E. coli* Nissle 1917 on antioxidant status in liver **a** catalase, **b** SOD activity, **c** lipid peroxidation, and **d** reduced glutathione levels. Values are expressed as mean ± SEM (n = 5–6 each group). ***$P \leq 0.001$, **$P \leq 0.01$ compared with fructose control; $^{\&\&\&}P \leq 0.001$ compared with iron-deficient control

ALT levels were increased considerably in the iron-deficient and fructose control groups as compared to the normal control, and the levels were restored to normal in the probiotic *Ec*N (pAN1)- and *Ec*N (pAN6)-fed groups, *Ec*N (pAN6) being more efficient (Fig. 6).

Effect of probiotic treatment on messenger RNA expression

The expression pattern of genes related to iron transport is depicted in Fig. 7. These genes showed relatively lower expression in iron-depleted control group as compared with the non-depleted control.

Histopathological analysis

Histopathological analysis 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. 8).

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

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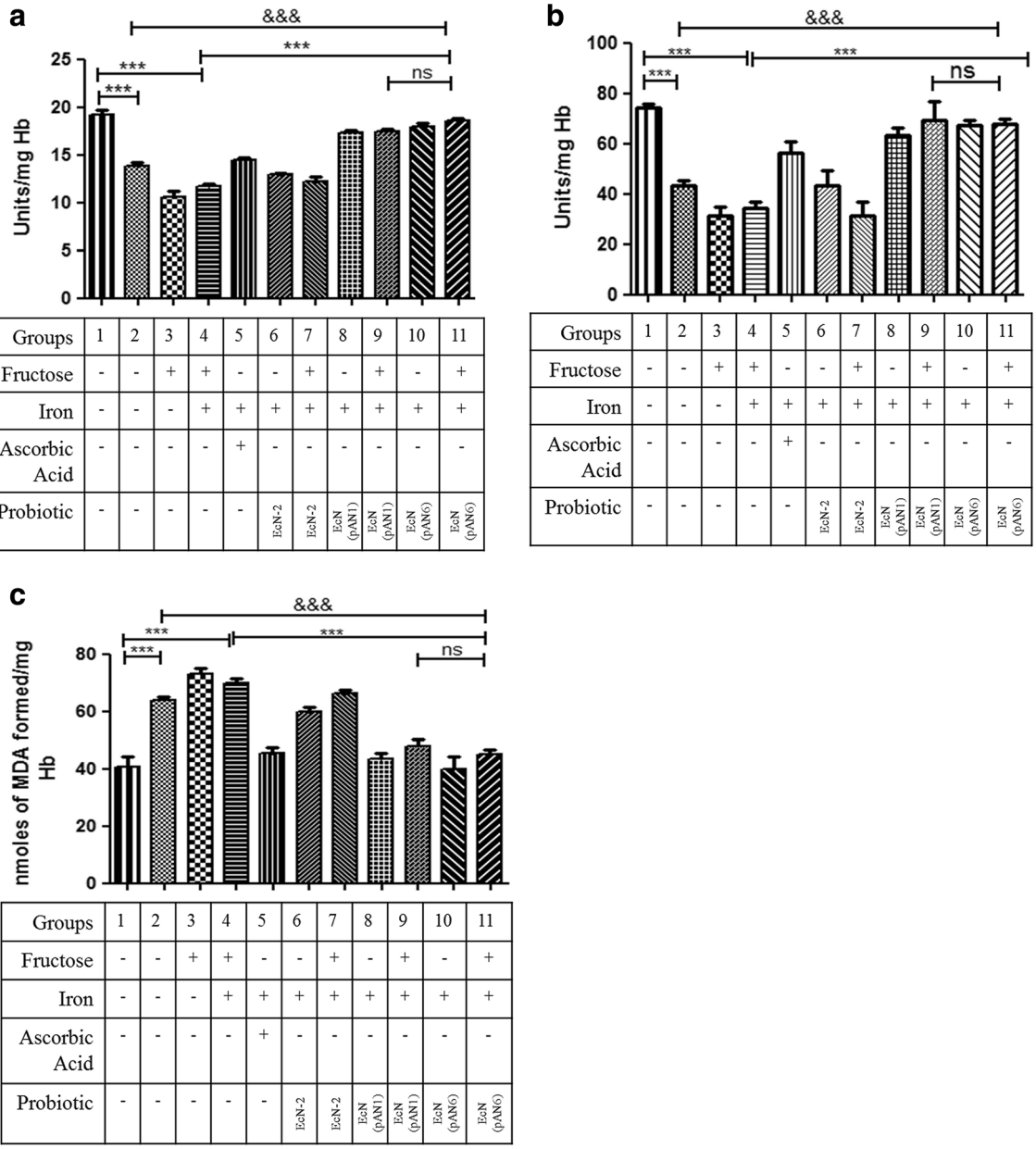

Fig. 5 Effect of genetically engineered probiotic *E. coli* Nissle 1917 on antioxidant status of blood. **a** Catalase. **b** SOD activity. **c** Lipid peroxidation. Values are expressed as mean ± SEM (n = 5–6 each group). $^{***}P \leq 0.001$ compared with fructose control, $^{\&\&\&}P \leq 0.001$ compared with iron-deficient control

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-deficient diet for 10 weeks decreased serum iron content by 57% as compared with non-depleted controls (Saini et al. 2014). One of the primary signs of iron deficiency is characterized by low serum iron and hemoglobin 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 abilities (Dell 1993). Fructose significantly increases iron-induced ferritin formation in human intestinal cell (Christides and Sharp 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 and Stoltzfus 2003). When rats were fed with fructose; ascorbic acid; and the *Ec*N-2, *Ec*N (*pqq*), and *Ec*N (*pqq-glf-mtl*K) probiotics after 10 weeks along with iron in the diet for 4 weeks, here also, Tf-

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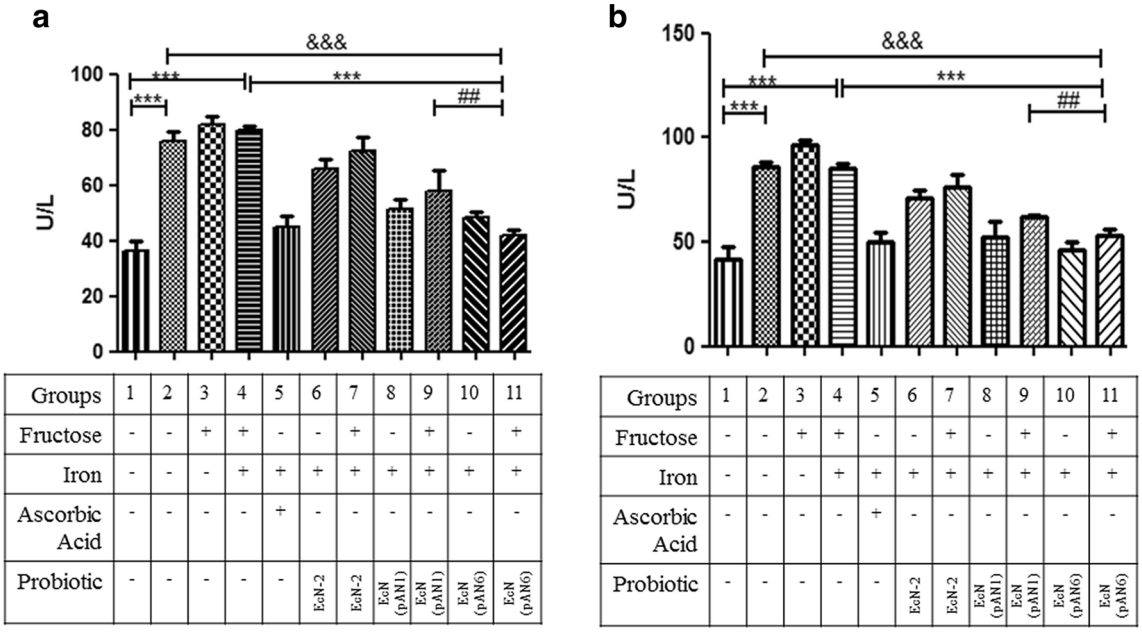

Fig. 6 Effect of genetically engineered probiotic *E. coli* Nissle 1917 on liver function markers. **a** AST. **b** ALT in serum. Values are expressed as mean ± SEM ($n = 5$–6 each group). ***$P \leq 0.001$ compared with fructose control, $^{\#\#}P \leq 0.01$ compared with fructose + iron + *Ec*N (pAN1), $^{\&\&\&}P \leq 0.001$ compared with iron-deficient control

bound Fe levels increased significantly by administration of fructose, ascorbic acid, *Ec*N (*pqq*), and *Ec*N (*pqq-glf-mtl*K). 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, or insulin sensitivity. Fructose can cause hypertriglycidemia and increase intrahepatic lipid levels (Tappy and Le 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 2012). 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

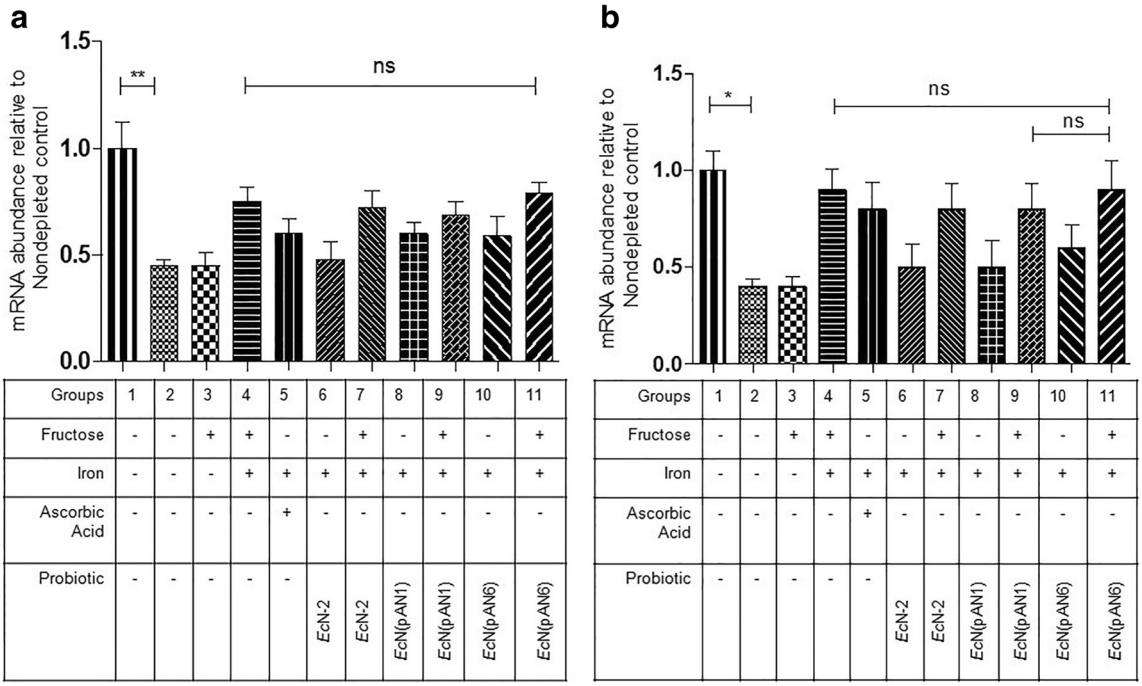

Fig. 7 Effect of iron-depleted diet and iron-repleted diet along with probiotic treatment on expression of iron transporter genes. **a** Transferrin. **b** Ferritin

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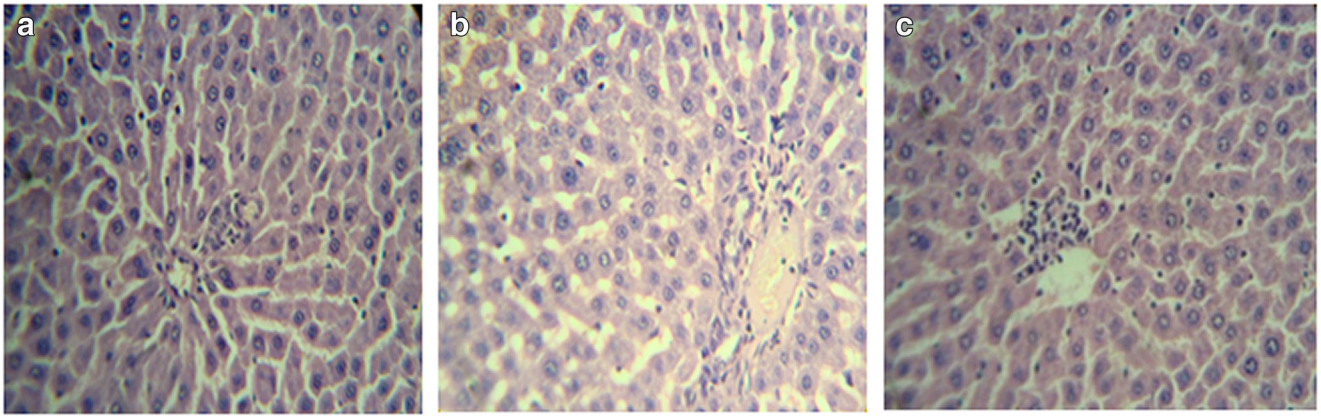

Fig. 8 Hematoxylin and eosin staining of rat liver tissue. Images were taken by Leica DME microscope at ×40 magnification. **a** Control. **b** Fructose. **c** *Ec*N (pAN6)

to almost normal values in the group that was fed with *Ec*N (*pqq-glf-mtl*K) and *Ec*N (*pqq*) and fructose, thus preventing the dyslipidemia and hyperglycemia that is caused due to fructose intake, with *Ec*N (*pqq-glf-mtl*K) showing more efficiency. This is supported by the fact that probiotic *Ec*N which act as a synbiotic in gut was effective in dealing with fructose-induced metabolic syndrome (Somabhai et al. 2016).

Fe-deficient rats show higher levels of oxidants and lipid peroxidation (Walter et al. 2002), and the iron-deficient patients show MDA levels significantly higher in contrast to the erythrocyte SOD, CAT activities, and GPx levels, which 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-mtl*K) and *Ec*N (*pqq*) groups. Probiotic *Ec*N secreting PQQ have demonstrated similar antioxidant effects against DMH-, rotenone-, alcohol-, metal-, and fructose-induced oxidative stress (Pandey et al. 2014; Singh et al. 2014; Singh et al. 2015; Raghuvanshi et al. 2016; Somabhai et al. 2016).

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-treated 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, in the present study, iron deficiency-induced liver damage 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-mtl*K), which can prevent the side effects of fructose consumption, could be a better strategy for enhancing iron absorption. Moreover, uptaken fructose is converted to mannitol by *Ec*N (*pqq-glf-mtl*K), which is a prebiotic and is fermented by the colonic microflora to produce SCFAs (Somabhai et al. 2016). 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, the present study demonstrates that supplementation of synbiotic along with fructose is an effective approach to deal with iron deficiency, which combines the beneficial effects of fructose, probioic *Ec*N (*pqq-glf-mtl*K), antioxidant PQQ, prebiotic mannitol, and SCFA.

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Compliance with ethical standards

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional guidelines for the care and use of animals were followed.

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1. "Targeting fructose induced disorders using genetically engineered *Escherichia coli* Nissle 1917 (*Ec*N) secreting pyrroloquinoline quinone and mannitol dehydrogenase". Archana Chaudhari, Ruma Raghuvanshi, Nareshkumar G. at Cell Symposia: "Human Immunity and the Microbiome in Health and Disease", Montreal, Canada.
2. "Genetically engineered probiotic *Escherichia coli* Nissle 1917 producing fructose dehydrogenase ameliorates fructose induced dietary disorder in rats". Chaudhari A. S , Raghuvanshi R., Nareshkumar G. at 2nd Annual Conference along with an International Symposium on "Probiotics and Microbiome: Gut and Beyond", New Delhi.
3. "Amelioration of Cadmium and Mercury induced liver and kidney damage in rats by genetically engineered probiotic *Escherichia coli* Nissle 1917 producing citric acid".Raghuvanshi R., Chaudhari A.S , Nareshkumar G. at 83rd Annual Meeting of Society of Biological Chemists (India) , "Evolution : Molecules to Life", Bhubaneswar.
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